EXCLI Journal 2005;4:130-140 – ISSN 1611-2156
Received: 22. October 2005, accepted: 21. November 2005, published 5. December 2005

Original article:

Increasing trend of multiple resistance and genomic mobility of *Neisseria gonorrhoeae* to penicillin and quinolone

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ABSTRACT

A significant decline of gonorrhea incidence has been observed during the years 1990-99. However, a slight increase in the number of cases has been reported in 2000. In addition, an increase in resistant strains has been found in Thailand. In this study, 207 isolates of *N. gonorrhoeae* from patients attending Bangrak hospital (National Centre of Sexually Transmitted Infections), 67 isolates obtained during January-March 2000, 74 isolates obtained during January-March 2002, and 66 isolates obtained during October-December 2002, were tested. All isolates were susceptible to ceftriaxone while 71.5% and 74.4% were resistant to penicillin and quinolone, respectively. The high level of ciprofloxacin resistance (MIC ≥4 mg/L) also increased from 13.4% during January-March 2000 to 25.8% during October-December 2002. Multiple resistance determinants commonly coexisted in a single isolate so that the level of resistance was increased. The incidence of double resistance determinants, penicillin and quinolone resistance, were significantly increased from 34.3% among isolates during January to March 2000 up to 77.3% among isolates during October to December 2002 ($P < 0.001$). In addition, an isolate obtained in 2002 resisted to spectinomycin with a high MIC (>1.024 g/L). Several plasmid patterns have been identified and various patterns of the plasmid can be artificially transferred and maintained their expression in *Escherichia coli* transformants. Such evidences infer the high mobility of resistant genome among microorganisms in the region. Moreover, the significant increase in penicillin and quinolone resistance herein, indicates the selective pressure and the diversity of genomic distribution among *N. gonorrhoeae* in Thailand. Primers JDA (5'-TAC TCA ATC GGT AAT TGG CTT C-3') and JDB (5'-CCA TAT CAC CGT CGG TAC TG-3') have been designed from sequences of the Asia, the Africa, and the Toronto β-lactamase plasmids. By using the JDA and the JDB as PCR primers, our data reveal the highest prevalence and a significantly increasing trend of the epidemic Africa type of genomic β–lactamase.

Keywords: *Neissseria gonorrhoeae*, antimicrobial susceptibility, quinolone resistance, penicillin resistance

INTRODUCTION

Gonorrhea remains one of the most common sexually transmitted infections (STIs) in developing countries, especially in South and Southeast Asia (Gerbase et al., 1998a; Gerbase et al., 1998b). Approximately 62 million cases of gonorrhea among the 340 million cases of curable sexually transmitted infections (gonorrhea, chlamydia, syphilis, and trichomoniasis) have occurred worldwide in 1999. The global health problem is concerned with the development of antimicrobial resistance of *N. gonorrhoeae* due to the wide dissemination of resistant clones and emerging of novel resistance (Knapp et al., 1997a). Southeast Asia is most likely an origin of drug resistance (Joesoef et al., 1994; Putnam et al., 1992). Penicillinase-producing strains have been firstly isolated in 1976 in Southeast Asia, and resistance to spectinomycin and tetracycline of gonococci has emerged in the 1980s. Fluoroquinolone-resistant gonococci have been found in several Asian countries during the early 1990s reaching the high level in Hong Kong, the Philippines, Japan, and Singapore (Knapp et al., 1997a; Putnam et al., 1992; Tanaka et al., 2000).

The third-generation cephalosporins, fluoroquinolones, and spectinomycin are included for the treatment recommendation of gonorrhea by the World Health Organization (WHO). They have been proven to have excellent efficacy while emergence of resistance remains a serious matter for combating the infection. The levels of gonococcal resistance to penicillins, tetracyclines, and fluoroquinolones have been frequently reported. The treatment has thus become more and more complicated particularly due to a multiple resistance. In China, the multiple resistances of gonococci to spectinomycin and ciprofloxacin have been reported (Li et al., 2000). This study is aimed to validate the antimicrobial susceptibilities of *N. gonorrhoeae* isolated in Thailand. Production of β-lactamase and genomic of the resistance are also unveiled among the gonococci.

MATERIALS AND METHODS

N. gonorrhoeae strains

A total of 207 clinical isolates of *N. gonorrhoeae*, 67 isolates obtained from January to March 2000, 74 isolates obtained from January to March 2002, and 66 isolates obtained from October to December 2002, were tested. All isolates of *N. gonorrhoeae* were collected from patients with gonorrhea attending the national center for sexually transmitted infection clinic in Bangrak hospital. *N. gonorrhoeae* was identified based on the appearance of Gram-negative diplococci, positive oxidase reaction, and sugar utilization patterns. The isolates were suspended in 10% skimmed milk with 15% glycerol and stored at -80^{o}C until further experiment. Cultivation was performed on chocolate agar (Gonococcal base medium supplemented with 1% bovine hemoglobin and 1% isovitalex) in a moist candle jar at 33-35^{o}C.

Beta-lactamase detection

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

Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) were determined by the E-test on a chocolate agar plate. Briefly, *N. gonorrhoeae* isolates were suspended in tryptic soy broth (TSB) to a turbidity equivalent to a 0.5 McFarland standard No.1. Chocolate agar plates were spread over the entire surface with a swab to deliver the bacterial suspension. The E-test strips containing an exponential antibiotic gradient on one side and an MIC reading scale marked on the other was firmly placed on the culture surface. The culture plate was then incubated in a moist candle jar for 24 h at 35°C. MICs were determined by recording of value at the point of intersection between the edge of the zone of growth inhibition and the strip. The antimicrobial agents tested were penicillin G,

spectinomycin, ceftriaxone, norfloxacin, ofloxacin, and ciprofloxacin. Interpretive criteria for susceptibility testing of all antimicrobial agents were used according to the National Committee for Clinical Laboratory Standards (NCCLS, 2004) except norfloxacin was followed the Centers for Disease Control and Prevention (CDC) (Knapp et al., 1997a). The MIC for interpretive results to penicillin, spectinomycin, norfloxacin, ofloxacin, and ciprofloxacin were ≤ 0.06 mg/L, ≤ 32 mg/L, ≤ 0.25 mg/L, ≤ 0.25 mg/L, and ≤ 0.06 mg/L for susceptible isolates while ≥ 2 mg/L, ≥ 128 mg/L, ≥ 1 mg/L, ≥ 2 mg/L, and ≥ 1 mg/L were for resistant isolates, respectively.

Isolation of plasmid DNA and transformation

Plasmid extraction

Plasmid DNA of *N. gonorrhoeae* was prepared by alkaline lysis method (Sambrook et al., 1990). Suspended DNA was treated with RNase A (final concentration 20 mg/L) before analyzed on 0.7% agarose gel electrophoresis.

Transformation

Escherichia coli strain TG1 was cultivated in LB medium and transformed with plasmid by using the conventional calcium chloride method (Sambrook et al., 1990). Transformants were selected on ampicillin plate and transferring plasmids were analyzed on 0.7% agarose gel electrophoresis. The restriction enzymes, *Hin*dIII, *Pst*I, *Eco*RI, *Bam*HI, were used as described by the manufacturer's instructions.

Susceptibility testing

Antimicrobial susceptibility was tested using a disc diffusion method for the transformants. The obtained ampicillin resistant transformants were tested against amoxicillin-clavulanate (20 μg/10 μg), chloramphenicol (30 μg), tetracycline (30 μg), sulfamethoxazole-trimethoprim (23.75 μg/1.25 μg), gentamicin (10 μg), norfloxacin (10 μg), and ciprofloxacin (5 μg).

PCR amplification of the β-lactamase producing plasmids

Primers JDA (5'-TAC TCA ATC GGT AAT TGG CTT C-3') and JDB (5'-CCA TAT CAC CGT CGG TAC TG-3') have been designed from sequences of the Asia, the Africa, and the Toronto β-lactamase plasmids by Dillon et al (1999). Whole cells were suspended in distilled water at a 0.5 McFarland standard No. 1 and used as a template. PCR amplification was performed in 25 μl of a reaction mixture which contained 2.5 μl of 10x PCR buffer containing 15 mM $MgCl_2$, 2.5 μl of each primers (10 pmol/μl), 2.5 μl of each of the four deoxyribonucleotide triphosphates (2 mM), 0.3 μl of *Taq* DNA polymerase (1 U/μl) (Fermentas), and 6 μl of template. After an initial incubation at 94°C for 10 min, 30 cycles were conducted with denaturing at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 5 min, with a final extension at 72°C for 10 min. A cell suspension of *E. coli* TG1 containing no gonococcal plasmids and non-penicillinase producing *Neisseria gonorrhoeae* were used as negative controls.

Statistical analysis

Data were analyzed by chi-square test (Eveillard et al., 2005). Statistical significance for all *P*-values was set at 0.05.

RESULTS

Trends of multiple drug resistance

Susceptibility to various antimicrobial agents of all together the 67 isolates obtained during January to March 2000, 74 isolates obtained during January to March 2002, and 66 isolates obtained during October to December 2002 were summarized in Table 1. Among the 207 isolates, only 22 (10.6%) isolates were susceptible to all antimicrobial agents including penicillin, ceftriaxone, norfloxacin, ofloxacin, ciprofloxacin, and spectinomycin. On the other hand, 148 (71.5%) isolates were resistant to penicillin (MIC, 2.0 μg/ml) and all were positive for β-

Table 1 Antibiotic susceptibility of 207 *N. gonorrhoeae* isolates from Bangkok during the period of January to March 2000, January to March 2002 and October to December 2002.

Antibiotics	Susceptibility (mg/L)								
	January-March 2000 (n=67)			January-March 2002 (n=74)			October-December 2002 (n=66)		
	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}
Penicillin	0.032->256	2	64	0.064->256	6	96	0.064->256	12	128
Norfloxacin	0.003-96	1	12	0.016-16	2	16	0.016-32	4	12
Ofloxacin	0.006->32	0.380	8	0.002-16	0.750	12	0.006-16	3	12
Ciprofloxacin	0.002->32	0.125	4	0.002-8	0.250	4	0.003-16	1	6
Spectinomycin	4-16	12	16	4->1,024	6	8	4-16	8	12
Ceftriaxone	<0.002-0.047	0.004	0.008	0.002-0.023	0.004	0.008	<0.002-0.032	0.004	0.008

lactamase production. The MIC_{50} ratios of penicillin, calculated by dividing the MIC_{50} for the isolates from 2002 by the MIC_{50} for the isolates from 2000, showed a 3-fold difference for January-March 2002 and a 6-fold difference for October-December 2002. For quinolone resistance, the MIC_{50} ratios also showed 2 to 8 fold differences. Figure 1 showed isolation rates per period of *N. gonorrhoeae* strains that were resistant and less susceptible to penicillin, norfloxacin, ofloxacin, ciprofloxacin, and spectinomycin. The number of ciprofloxacin, ofloxacin and norfloxacin-resistant strains were increased from 28%, 28% and 64% during January-March 2000 to 56%, 68% and 86% during October-December 2002, respectively. These differences were statistically significant (P < 0.005). With the exception of 2 strains, all ciprofloxacin-resistant strains were also found to be ofloxacin-resistant (n = 83). In addition, there was a positive correlation

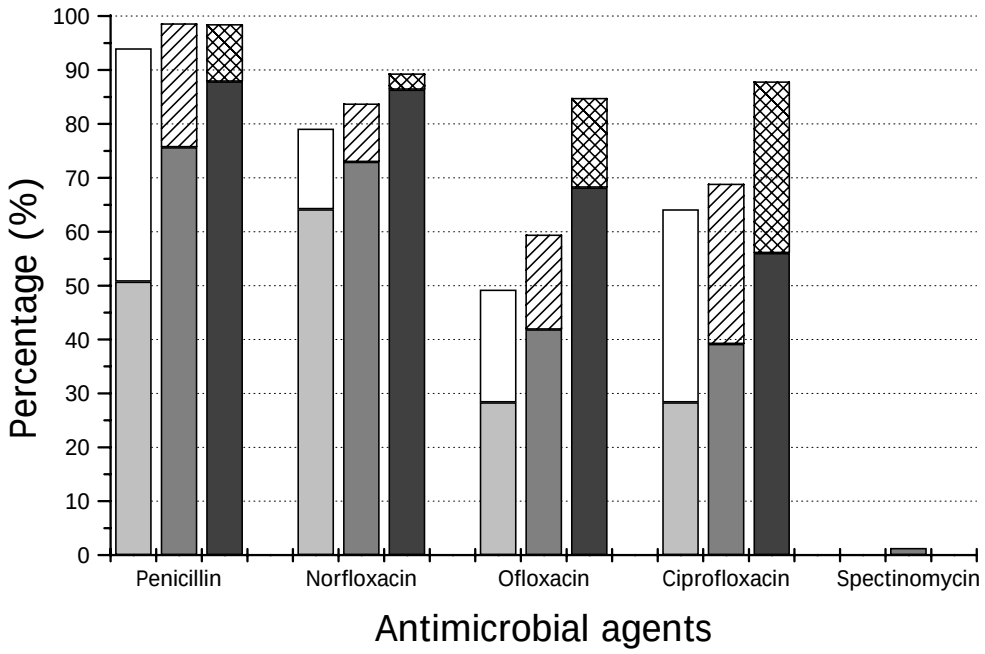

Figure 1: Percentage of *Neisseria gonorrhoeae* isolates from Bangrak hospital, Bangkok, in each period that were resistant and less susceptible to penicillin, quinolones (norfloxacin, ofloxacin, ciprofloxacin), and spectinomycin.

Percent Resistance in 2000 (Jan-Mar)
Percent Resistance in 2002 (Jan-Mar)
Percent Resistance in 2002 (Oct-Dec)
Less susceptible in 2000 (Jan-Mar)
Less susceptible in 2002 (Jan-Mar)
Less susceptible in 2002 (Oct-Dec)

between the susceptibility to ciprofloxacin and ofloxacin in all strains tested (r^2= 0.93). The high level of ciprofloxacin resistance (MIC ≥ 4 mg/L) was also found increased from 13.4% during January-March 2000 up to 20.3% and 25.8% during January-March 2002 and during October-December 2002, respectively. In addition, multiple resistance determinants coexisted in a single isolate were significantly found in 2002 (Table 2). The prevalence of double resistance determinants, penicillin and quinolone resistance, were significantly increased from 34.3% among isolates during January-March 2000 to 58.1% among isolates during January-March 2002 (P < 0.01) and 77.3% among isolates during October-December 2002 (P < 0.001). Moreover, during January-March 2002 one isolate was found resisting to spectinomycin with a very high MIC of > 1.024 g/L.

Ampicillin resistance and genomic type of β–lactamase

Elevated trend of penicillin resistance was observed. The PPNG isolates increased significantly from 50.7% during January-March 2000 to 75.7% during January-March 2002 (P = 0.002) and 87.9% during October-December 2002 (P < 0.001). By using the JDA and the JDB primers, all 167 PPNG isolates except one were identified to carry β–lactamase plasmids by PCR. This was confirmed by *Hin*fI restriction enzyme digestion. The amplified products of 4.9, 3.1 and 2.6 kb were revealed. The band sizes of amplicon were corresponding to the Asia, the Africa and the Toronto/Rio-type plasmids, respectively (Figure 2). The Asia type plasmid was found in 24 (14.5%) isolates while 101 (60.8%) isolates contained the Africa type plasmid and 41 (24.7%) isolates contained the Toronto type plasmid. These epidemic plasmids were reported in each period with different percentages. The Africa type was increased significantly from 24.2% during January-March 2000 to 61.4% during January-March 2002 (P < 0.001) and 76.3% during October-December 2002 (P < 0.0001). On the other hand, the Asia type and the Toronto type were markedly decreased from 27.3% and 48.5% during January-March 2000 to 3.9% and 19.7% during October-December 2002 (Figure 3). Interestingly, the Africa type plasmid carrying isolates showed lower penicillin MIC comparing to the Asia and the Toronto type plasmid carrying isolates (Figure 4).

Table 2 Distribution of antibiotic resistance and co-resistant among *N. gonorrhoeae* isolated from patients at various time periods.

Drug resistances	January-March 2000 (n=67)	January-March 2002 (n=74)	October-December 2002 (n=66)
Susceptible	**13 (19.4%)**	**7 (9.5%)**	**2 (3.0%)**
Penicillinase producing *N. gonorrhoeae* (PPNG)	**11 (16.4%)**	**13 (17.6%)**	**7 (10.6%)**
Quinolone resistances	**20 (29.9%)**	**11 (14.9%)**	**6 (9.1%)**
Norfloxacin resistance	20 (29.9)	11 (14.9)	6 (9.1)
Ofloxacin resistance	7 (10.4)	3 (4.1)	5 (7.6)
Ciprofloxacin resistance	7 (10.4)	3 (4.1)	5 (7.6)
PPNG and Quinolone resistances	**23 (34.3%)**	**42 (56.8%)**	**51 (77.3%)**
PPNG and Norfloxacin resistance	23 (34.3)	42 (56.8)	51 (77.3)
PPNG and Ofloxacin resistance	12 (17.9)	28 (37.8)	40 (60.6)
PPNG and Ciprofloxacin resistance	12 (17.9)	26 (35.1)	32 (48.5)
PPNG, Norfloxacin and Spectinomycin resistance	**0 (0.0%)**	**1 (1.4%)**	**0 (0.0%)**

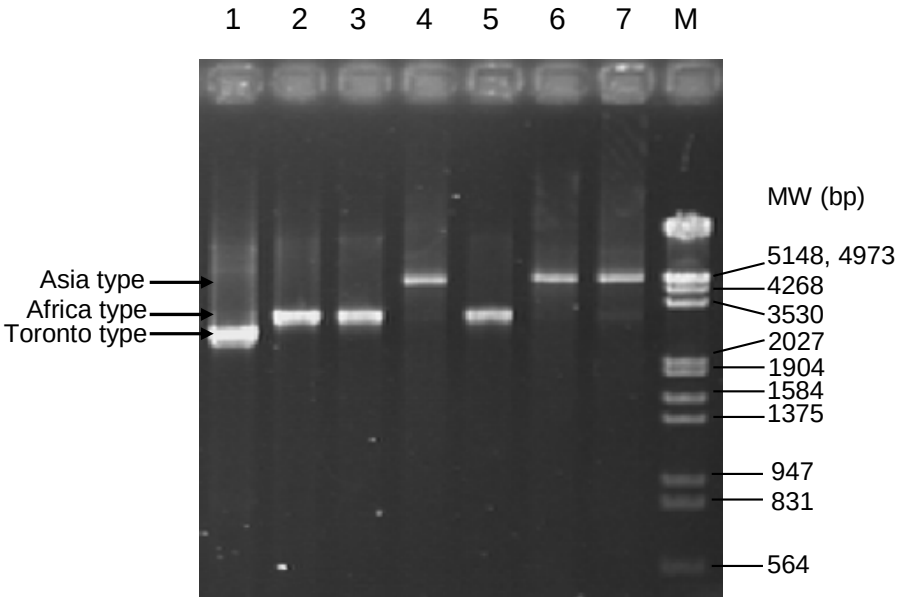

Figure 2: Gel representative of PCR amplicons of β–lactamase producing genomic types from *Neisseria gonorrhoeae* isolates. Lane 1 represented product of the Toronto type, lane 2, 3, and 5 represented product of the Africa type and 4, 6-7 represented product of the Asia type. Lane M is a lambda *Hin*d III/*Eco*R I fragments.

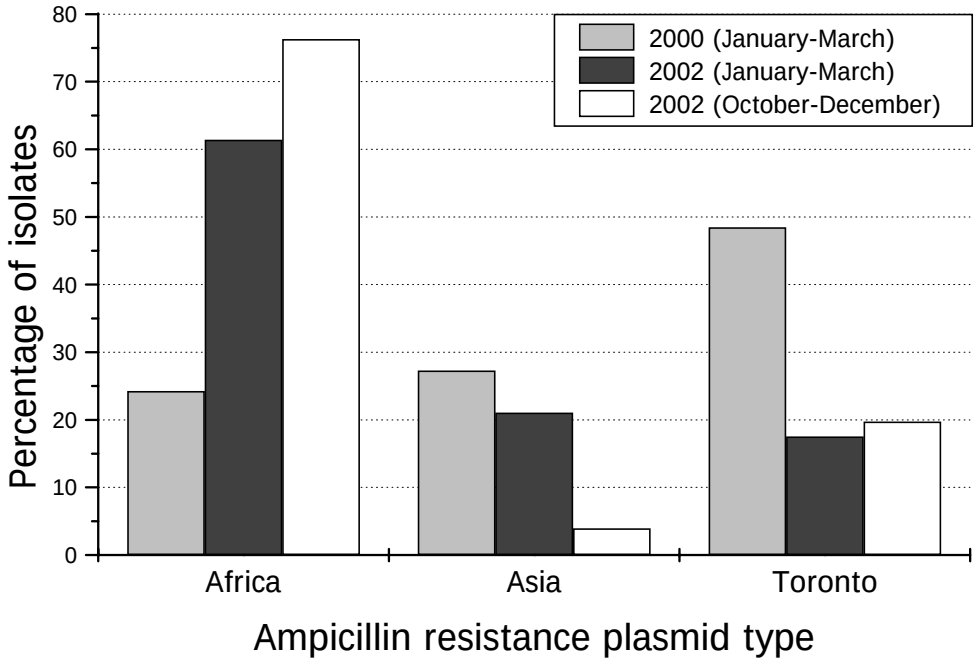

Figure 3: Mobility of Penicillinase producing *Neisseria gonorrhoeae* (PPNG) distributed among types of β-lactamase from isolates obtained from patients of Bangrak hospital, Bangkok at different time frame.

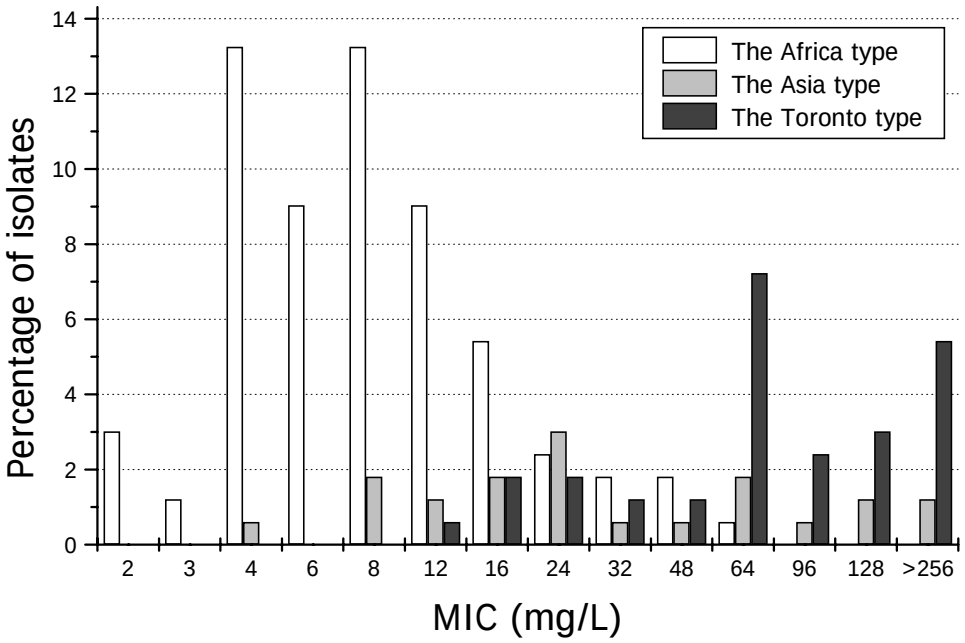

Figure 4: Corresponding of genomic resistance types and MICs in penicillinase producing *Neisseria gonorrhoeae* (PPNG) isolates from patients of Bangrak hospital, Bangkok.

Furthermore, the Asia, the Africa, and the Toronto type plasmids could be transferred and continuously expressed in *E. coli.*

Transferable antibiotic resistance plasmids

All 207 *N. gonorrhoeae* isolates carried plasmids that could be purified by alkaline lysis methods. The plasmids from individual organism were classified into patterns and all patterns remained their unique self-individual patterns after digestion with four restriction enzymes, *Pst*I, *Eco*RI, *Hin*dIII, and *Bam*HI. There was no significant difference in plasmid profiles between the strains in each antimicrobial susceptibility pattern. By randomly selection, we found that plasmid profiles of penicillin resistant isolates from each pattern could be transferred by artificial transformation and expressing ampicillin resistance in *E. coli* transformants. Although multiple resistance determinants were detected in *N. gonorrhoeae,* transformants did not show the co-resistance determinants with any kinds of antimicrobials (chloramphenicol, tetracycline, sulfamethoxazole-trimethoprim, gentamicin, norfloxacin, or ciprofloxacin). In addition, production of β–lactamase in transformants was inhibited by clavulanic acid.

Ampicillin resistance plasmids were successfully maintained in the *E. coli* host under ampicillin selective pressure. The plasmid profiles from ampicillin resistance *E. coli* transformants were classified into three patterns and the diversity of plasmids was identified (Figure 5). We found that these three types of plasmid could be Asia, Africa, and Toronto types after digestion with the restriction enzymes *Pst*I, *Eco*RI, *Hin*dIII, and *Bam*HI. Using primers JDA and JDB, we revealed the same plasmid types as in the original *N. gonorrhoeae* isolates (Figure 2).

DICUSSION

Herein, we report a high prevalence of resistant strains in Thailand. In 2002, the penicillin and ciprofloxacin resistance isolates has been found to be 81% and 47%, respectively. This result is agreed with the report of the WHO Western Pacific Gonococcal Antimicrobial Surveillance Programme (WHO, 2002). Generally, the prevalence of both penicillin and quinolone

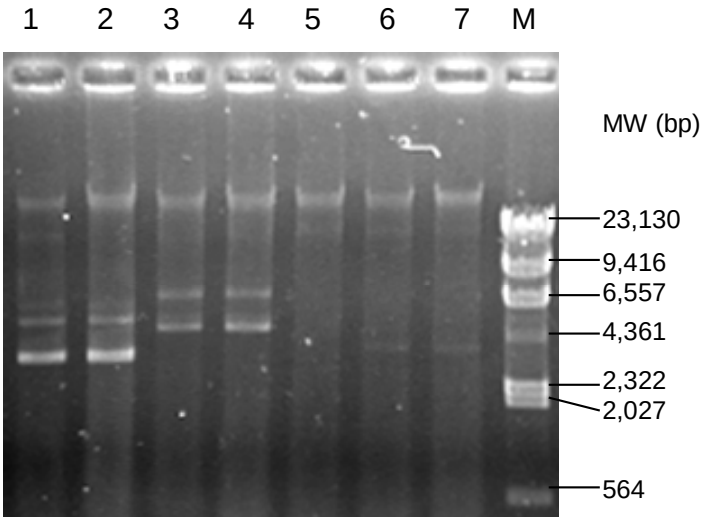

Figure 5: Gel representative of plasmid profiles of ampicillin resistance transformants. Lane 1-2 represented pattern I, lane 3-4 represented pattern II, lane 5-7 represented pattern III, and lane M is a Lambda *Hin*d III fragments.

resistance are high in Southeast Asia and some of the Asian countries. This has been supported by the reports in Laos (100%), the Philippines (92%), Malaysia (60%), Brunei (60%), Singapore (55%), and Vietnam (30%). For quinolone resistance, a very high proportion of QRNG (Quinolone resistance *Neisseria gonorrhoeae*) was found in Korea (95%), Hong Kong (94%), China (93%), Japan (84%), Vietnam (79%), Laos (76%), Brunei (64%), the Philippines (60%), Singapore (51%), and Malaysia (50%). Most of the QRNG in these countries display high-level drug resistance. These results are different from the isolates from Australia and New Zealand in which less than 20% of them are penicillin or quinolone resistance. We also find the elevated trend of antimicrobial resistance during 2000-2002. This is agreed with several recent reports on gonococci from many regions. Moodley and Strum (2005) have reported the increasing trend of ciprofloxacin resistance in South Africa during 2002-2005 while the QRNG has been found increasing in many global regions (Martin et al., 2005; Katz et al., 2003; Ray et al., 2005). Emergence and spread of QRNG may have been accelerated by heavy use of the quinolones. Although, penicillin and quinolones are no longer recommended for the treatment of gonorrhea in Southeast Asian countries, application of both drugs on the treatment of unrelated infections may continue to exert selective pressure on gonococcal strains.

Unlike penicillin and quinolones, spectinomycin and ceftriaxone continue to be useful for the treatment of gonorrhea in many geographical areas including Thailand. There is no reported case of treatment failure with the third generation cephalosporin. However, we report a spectinomycin resistance isolate in 2002 with a very high MIC. Spectinomycin resistant isolates have sporadically been found in many regions. The resistance to spectinomycin usually occurs by chromosomal mutation resulting in high level resistance. In Southeast Asia, Clendennen et al (1992a) have reported since 1989 that 8.5% of 117 isolates from the Philippines are resistant to spectinomycin. This finding is corresponded to the observation from Thailand in 1990 (Clendennen et al., 1992b). Therefore, it is important to monitor the drug susceptibility testing as well as the clinical efficacy of this regimen since spectinomycin is commonly used in Thailand.

Resistance to penicillin can be mediated both by chromosomal change to effect the penicillin binding proteins (PBPs) and by production of β–lactamase via episome. Both

chromosomally mediated resistance (CMRNG) and penicillinase-mediated resistance (PPNG) are widespread. The increase on prevalence of penicillin resistance from our findings reveals a steady increasing trend of the resistance in Thailand. Penicillin resistance isolates have been reported to be 64.9% in 1990 (Clendennen et al., 1992b), 77.2% during 1994-1995 (Knapp et al., 1997c), 50.7% in January-March 2000 (this study), 75.7% in January-March 2002 (this study) and 87.9% in October-December 2002 (this study).

Several kinds of ampicillin resistance plasmids have been found in *N. gonorrhoeae* such as the Asia (7426 bp), the Africa (5599 bp), the Toronto (5154 bp), the Rio (5154 bp), the Nymes (6798 bp) and the New Zealand (9309 bp) types. However, the Asia, the Africa, and the Toronto type plasmids have been associated with epidemic outbreaks while the others have been isolated sporadically. Prevalence of each plasmid type is varied depending on geographic location. Knapp et al (1997b) have reported in Manila in 1994 that PPNG accounts for 66 (93%) of 71 penicillin resistance isolates. Among these PPNG, the Asia, the Africa, and the Toronto type β-lactamase plasmids represent for 18.2%, 68.2%, and 12.1%, respectively. In Thailand, the Africa type plasmid is on the increasing trend from 3.8% during 1994-1995 (Knapp et al., 1997c) to 24.2% in 2000 (this study) and 69.9% in 2002 (this study). While the Asia type plasmid shows a declining trend from 96.2% during 1994-1995 (Knapp et al., 1997c) to 27.3% in 2000 (this study) and 11.3% in 2002 (this study). The Toronto type plasmid also shows decreasing from 48.5% in 2000 (this study) to 18.8% in 2002 (this study). In addition, all three kinds of epidemiologically penicillin resistance plasmids of *N. gonorrhoeae* can be transferred by artificial transformation and expressed in *E. coli.* This has also been reported in the cases of *Haemophilus ducreyi* (Prachayasittikul et al., 2000). Several groups have proposed that β–lactamase-producing plasmids of gonococci may possibly originate from a *H. ducreyi* host via transformation involving the Asia and the Africa progenitor (Roberts, 1989).

Our findings also reveal the significant increase on the prevalence of multiple resistance determinants (penicillin and quinolones) coexisting in a single isolate. However, quinolone resistance gene can not be co-transferred with ampicillin resistance plasmid to *E. coli*. In general, quinolone resistance involves chromosomal mutation that modifies the target DNA gyrase (or DNA topoisomerase IV) or accelerates the efflux to decrease quinolone accumulation. There are only sporadic reports of plasmid-mediated quinolone resistance (Piddock, 1999). However, multiple resistance plasmid transferring has been reported in the case of *Klebsiella pneumoniae* to other Enterobacteriaceae and *Pseudomonas aeruginosa* (Martinez-Martinez et al., 1998).

In summary, *N. gonorrhoeae* strains in Thailand belong to a variety of penicillin/quinolone resistance phenotypes. A significant increase on the prevalence of multiple resistance determinants (penicillin and quinolones) coexisting in a single isolate is also reported. All penicillin resistance isolates produce β–lactamase by carrying one of the epidemic β–lactamase plasmids of the Asia, the Africa, or the Toronto type. However, the diversity of genomic distribution has been found. The Africa plasmid type is the most prevalence and being on the increasing trend. Emergence and spread of antibiotic resistance may have been partially due to the widely use of the drugs for the treatment of gonorrhoeae and other diseases. Penicillin and quinolones are no longer recommended for the treatment of gonorrhea in Southeast Asian countries although ciprofloxacin is still recommended by the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC). These data indicate that antimicrobial resistance is an important factor when considering the choice of agents for the treatment of gonococcal infections. In addition, the gonococcal β–lactamase producing plasmids can be used as a marker

for epidemiologically benefit along with the susceptibility pattern. Furthermore, the mobility of the episomes infers the high tendency of drug resistance development among infectious microorganisms in this region. The assessment of the antimicrobial resistance is of great importance to monitor the emergence and spread of resistance and planning of appropriate treatment regimens.

Acknowledgements: R.L. was partially supported by the grant for promoting young researcher from Mahidol University. Some arrangements of this manuscript were generously provided by the help of Dr. Tanawut Tantimongcolwat and Asst. Prof. Dr. Chartchalerm Isarankura Na Ayudhya.

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