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ภาคผนวก

ผลงานที่ได้จากโครงการวิจัยที่ได้รับทุน

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

1.1 Lawung R, Cherdtrakulkiat R, Charoenwatanachokchai A, Nabu S, Suksaluk W, Prachayasittikul V. One-step PCR for the identi.cation of multiple antimicrobial resistance in *Neisseria gonorrhoeae.* Journal of Microbiological Methods 2009; 77: 323–325.

2. การนำผลงานวิจัยไปใช้ประโยชน์ (เชิงวิชาการ)

2.1 ฝึกอบรมนานาชาติหลักสูตร "International Training Course on STI/HIV Laboratory Diagnosis" ในหัวข้อเรื่อง Present and future identification for *Neisseria gonorrhoeae* และ Drug resistance of *Neisseria gonorrhoeae* ในวันที่ 26 กรกฎาคม 2550 ณ กลุ่มโรคติดต่อทางเพศสัมพันธ์ เขตบางรัก กรุงเทพฯ

2.2 ฝึกอบรมนานาชาติหลักสูตร "Current and future trend in gonorrhoea" ในวันที่ 21 สิงหาคม 2551 ณ กลุ่มโรคติดต่อทางเพศสัมพันธ์ เขตบางรัก กรุงเทพฯ

2.3 ฝึกอบรมนานาชาติหลักสูตร "International Training Course on STI/HIV Laboratory Diagnosis" ในหัวข้อเรื่อง Present and future identification for *Neisseria gonorrhoeae* และ Drug resistance of *Neisseria gonorrhoeae* ในวันที่ 24 กันยายน 2552 ณ กลุ่มโรคติดต่อทางเพศสัมพันธ์ เขตบางรัก กรุงเทพฯ

3. อื่น ๆ

3.1 เสนอผลงานในที่ประชุมวิชาการระดับนานาชาติ เรื่อง Ratana Lawung, Sunanta Nabu, Wanwisa Suksaluk, Prasong Sirivongrangson, Virapong Prachayasittikul. Genotypic diagnosis of multiple drug resistance of *Neisseria gonorrhoeae*. The VI Princess Chulabhorn International Science Congress, 25-29 November 2007 Bangkok.

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Note

One-step PCR for the identification of multiple antimicrobial resistance in *Neisseria gonorrhoeae*

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ABSTRACT

One-step multiplex PCR was developed for the identification of gonococci and antimicrobial-resistant profiles. From forty *Neisseria gonorrhoeae* isolates, the penicillinase-producing *N. gonorrhoeae* (PPNG), the high-level tetracycline-resistant *N. gonorrhoeae* (TRNG), and the ciprofloxacin-resistant *N. gonorrhoeae* (CRNG) were successfully classified. Our method provides expediency and benefit to epidemiology and antimicrobial-resistance mobility with 100% sensitivity and specificity for gonococcal-detection. The detection limit was 500 CFU/reaction.

A significant increase in multiple resistance of *Neisseria gonorrhoeae* toward penicillin, tetracycline and fluoroquinolones has been reported worldwide (Gerbase et al., 1998; Knapp et al., 1997). The need to identify and characterize the resistance profile of *N. gonorrhoeae* has been recognized as a public health priority. This has been addressed by World Health Organization (WHO) in their establishment of surveillance programs for monitoring the resistance of *N. gonorrhoeae* to penicillin, tetracycline and ciprofloxacin. In Thailand, plasmid-mediated resistance to penicillin and tetracycline is commonly found among *N. gonorrhoeae* isolates. Of the six types of β-lactamase producing plasmids (Asia, Africa, Toronto, Rio, Nimes and New Zealand), the first three types have been associated with the spread of penicillin-resistant *N. gonorrhoeae* (PPNG) (Pagotto et al., 2000). High-level of tetracycline-resistant *N. gonorrhoeae* (TRNG) has been observed due to the acquisition of *tetM* gene, which has two determinants: American and Dutch, as carried on 25.2 MDa conjugative plasmid (Gascoyne-Binzi et al., 1993). Fluoroquinolone resistance is caused by mutation of *gyrA* and *parC*, where mutation at Ser-91 of GyrA is the most frequently reported among the fluoroquinolone-resistant isolates (Karunakaran and Sam, 2007; Tanaka et al., 2000). The presence of *Hin*fI restriction site at *gyrA* (Ser-91) makes it amenable for detection of Ser-91 mutation through the utilization of *Hin*fI restriction fragment length polymorphism of the *gyrA* (Deguchi et al., 1996).

In this study, we have developed a multiplex PCR method to identify gonococci and discriminate plasmid types of penicillinase-producing (Asia, Africa and Toronto), tetracycline-resistance (American and Dutch), and the decreased-susceptibility to ciprofloxacin. Disk diffusion and minimum inhibition concentration (MIC) have been included for comparison with our proposed approach.

Forty *N. gonorrhoeae* isolates from patients attending the National Center of Sexually Transmitted Diseases, Bangrak Hospital, Bangkok, Thailand in 2000 were selected as representative of various antimicrobial profiles including susceptible, mono-, double- and triple-resistance to penicillin, tetracycline and ciprofloxacin. Production of β-lactamase was detected by chromogenic cephalosporin test (Nitrocefin, Oxiod, UK) while susceptibility testing of penicillin, tetracycline and ciprofloxacin were performed according to the criteria of (Clinical and Laboratory Standard Institute, 2006).

GC1F, GC2F, GC3R and GC4R primers were designed to be complementary to the sequences of Asia, Africa and Toronto-types of β-lactamase plasmids (accession numbers U20374, U20375 and U20419, respectively) while TetMF and TetMR primers were designed from the sequences of American and Dutch-types of *tetM* plasmids (accession numbers L12241 and L12242, respectively). GyrAF and GyrAR primers were designed to amplify the portion of *gyrA* from the quinolone resistance-determining region (QRDR) (Tanaka et al., 2000). Purified plasmid of green fluorescent protein (pGFPuv) (Crameri et al., 1996) were used as positive control for PCR

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Table 1
Primer sequences, target genes and PCR amplicon sizes.

Primer names[a]	Primer sequences (5′-3′)	Target genes	Types	PCR amplicon sizes (bp)/*Hin*fI		References
				Before	After	
GC1F	AACTCACGGACAAAATCACGG	β-lactamase	Africa	1070	1070	This study
GC2F	CACCTATAAATCTCGCAAGCC	Producing	Asia	737	737	
GC3R	AACGCAAGCAGGACGAAATC	Plasmid	Toronto	435	435	
GC4R	CCTCCACCTTCATCCTCAGC					
TetMF	ACTGTTGAACCGAGYAAACCT	*tetM*	American	841	748 + 93	This study
TetMR	TCTATCCGACTATTTGGACGACG		Dutch	841	572 + 176 + 93	
GyrAF	CGGCGCGTACTGTACGCGATGCA	Gyrase A	Ser-91 mutation	278	278	Tanaka et al. (2000)
GyrAR	ATGTCTGCCAGCATTTCATGTGAGA		Non-mutation	278	166 + 112	
GFPuvF	GTCAGTGGAGAGGGTGAAGG	pGFPuv	GFPuv	571	394 + 177	This study
GFPuvR	ACCATGTGGTCACGCTTTTC					

[a] F: forward primer; R: reverse primer.

amplification using GFPuvF and GFPuvR as primers. PCR reaction was carried out using all five primer sets (Table 1). Approximately 10^8 CFU/ml of bacterial suspension (0.5 McFarland standard) was boiled for 15 min and used as DNA templates. Multiplex PCR was carried out in 20 μl of a reaction mixture containing 1x PCR buffer with 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 0.3 U Taq DNA polymerase (Fermentas, California, USA), 1 pmol GC1F primer, 0.1 pmol GC2F primer, 1 pmol GC3R primer, 0.1 pmol GC4R primer, 0.75 pmol TetMF primer, 0.75 pmol TetMR primer, 0.1 pmol GyrAF primer, 0.1 pmol GyrAR primer, 0.075 pmol GFPuvF primer, 0.075 pmol GFPuvR primer, 1 ng pGFPuv template, and 5 μl DNA template.

PCR amplification was performed in a Bio-Rad iCycler (BioRad, California, USA) under the following conditions: one cycle at 94 °C for 5 min, then 35 cycles at 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min 30 s and a final elongation step was performed 1 cycle at 72 °C for 10 min. PCR amplicons were digested with *Hin*fI (Fermentas, California, USA) according to manufacturer's recommendation. Various sizes of amplicons and restriction fragments were determined by 1.5% agarose gel electrophoresis. The sensitivity and specificity of our novel mutiplex PCR approach were evaluated using a panel of 40 *N. gonorrhoeae* isolates, 3 non-gonococcal *Neisseria* species and 39 non-*Neisseria* species as templates (Table 2). The detection limit was assessed using ten-fold dilutions of bacterial suspension at various concentrations from 10^6 to 10^1 CFU/ml (confirmed by the colony counting).

Using five primer sets, we were successful to identify gonococci and antimicrobial-resistant patterns. In this study, the 278 bp amplicon of *gyrA* (gonococcal detection marker) was detected in all 40 gonococcal isolates while absent in 42 non-gonococcal isolates. Therefore, both sensitivity and specificity of this method for gonococcal identification were found to be 100%. The gene amplification results showed 100% correlation with phenotypic-susceptibility profiles (Table 3). The proposed one-step PCR method distinguished between 31 PPNG and 9 non-PPNG isolates. Particularly, all β-lactamase plasmid types (Africa, Asia and Toronto) from PPNG isolates were classified with PCR amplicons sizes of 1070, 737 and 435 bp, respectively. The *tetM* plasmid and the *gyrA* were shown amplicon sizes of 842 and 278 bp, respectively. After *Hin*fI digestion, two types of *tetM* plasmids (American and Dutch) and the presence of Ser-91 mutation in *gyrA* were identification. Additionally, the amplicon size of the internal control (GFPuv) was shown to be 571 bp while digestion with *Hin*fI resulted in two fragments (394 and 177 bp) (Fig. 1). Moreover, all 16 non-gonococcal isolates that produced β-lactamase were negative for specific amplicon representing β-lactamase plasmid of *N. gonorrhoeae* while β-lactamase producers among *Haemophilus ducreyi* (5 isolates) gave the amplicons of approximately 1100 and 850 bp which were corresponded to Africa-type of β-lactamase plasmid and *tetM* plasmid, respectively. Such observation may attribute to genetic mobility between gonococci and *H. ducreyi* (Ison et al., 1998; McNicol et al., 1986; Prachayasittikul et al., 2000). Detection limit of this method was found to be 500 CFU/reaction. However, the sensitivity was found to increase up to 100-fold by separating the reaction into two separate tubes for the detection of (i) ampicillin- and tetracycline-resistant genes and (ii) *gyrA* and GFPuv genes.

Herein, we present a simple and rapid one-step PCR approach for simultaneous identification of gonococci along with their antimicrobial resistance profiles. The proposed method offers high potential for further application in the identification of gonococci directly from

Table 2
List of Bacteria strains isolated from clinical specimens.

Bacterial names	Number of isolates
1. *Neisseria gonorrhoea*	40
2. *N. meningitidis*	1
3. *N. sicca*	1
4. *N. mucosa*	1
5. *Haemophilus ducreyi*	6
6. *H. influenzae*	3
7. *Moraxella catarrhalis*	1
8. *Micrococcus lutens*	1
9. *Staphylococcus aureus*	1
10. *S. epiderimidis*	1
11. Group A *Streptococcus*	1
12. Group B *Streptococcus*	1
13. Group D *Streptococcus*	1
14. *Enterococcus faecalis*	1
15. *Bacillus subtilis*	1
16. *Corynebacterium diphtheriae*	1
17. *Listeria monocytogenes*	1
18. *Escherichia coli*	1
19. *Klebsiella pneumoniae*	1
20. *Salmonella chloleraesuis*	1
21. *Serratia marcesens*	1
22. *Shigella dysenteriae*	1
23. *Citrobacter freundii*	1
24. *Enterobacter cloacae*	1
25. *Morganella morganii*	1
26. *Yersinia enterocolitica*	1
27. *Plesiomonas shigelloides*	1
28. *Vibrio chlorera*	1
29. *V. mimicus*	1
30. *Aeromonas carviae*	1
31. *A. hydrophila*	1
32. *Achromobacter xylosoxidans*	1
33. *Pseudomonas aeruginosa*	1
34. *Burkholderia cepacia*	1
35. *Stenotrophomonas maltophilia*	1
36. *Acinetobacter haemolyticus*	1

Table 3
Comparison of phenotypic and genotypic characteristics of *N. gonorrhoeae* isolates as determined by multiplex PCR.

Phenotypic characteristics	Number of isolates	Penicillin				Tetracycline			Ciprofloxacin	
		Non-PPNG	PPNG			Non-TRNG	TRNG		Ser-91 of GyrA	
			Africa	Asia	Toronto		American	Dutch	Non-mutation	Mutation
1. Susceptible strains	2	2	–	–	–	2	–	–	2	–
2. Mono-drug resistance										
PPNG[a]	3	–	1	–	2	3	–	–	3	–
TRNG[b]	1	1	–	–	–	–	–	1	1	–
CRNG[c]	2	2	–	–	–	2	–	–	–	2
3. Double-drug resistance										
PPNG/TRNG	4	–	2	2	–	–	1	3	4	–
PPNG/CRNG	7	–	1	2	4	7	–	–	–	7
TRNG/CRNG	4	4	–	–	–	–	2	2	–	4
4. Triple-drug resistance										
PPNG/TRNG/CRNG	17	–	10	4	3	–	10	7	–	17
Total	40	9	14	8	9	14	13	13	10	30

[a] PPNG: penicillinase-producing *N. gonorrhoeae*.
[b] TRNG: tetracycline-resistant *N. gonorrhoeae*.
[c] CRNG: ciprofloxacin-resistant *N. gonorrhoeae*.

clinical specimens which would be of great benefit for the global population. Aside from evaluating antimicrobial susceptibility at the phenotypic level, which is typically afforded by conventional approaches, our proposed method provides the advantage of being able to assess antimicrobial susceptibility at the genotypic level as well. Therefore, the devised method is helpful for epidemiological applications as well as useful for mobility studies of antibiotic resistance gene among sexually transmitted pathogens.

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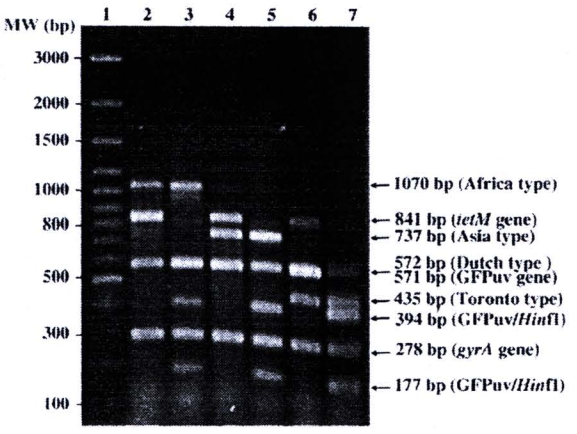

Fig. 1. PCR amplicons from one-step PCR system Agarose gel electrophoresis of PCR amplicons from *N. gonorrhoeae* isolates. Lane 1 represented 100 bp DNA Ladder markers. Lane 2, 4, 6 represented PCR amplicons of PPNG (Africa type) and TRNG (Dutch type), PPNG (Asia type) and TRNG (Dutch type), and PPNG (Toronto type) and TRNG (Dutch type). Lane 3, 5, 7 represented amplicons after digested with HinfI enzyme of sample in lane 2, 4, and 6, respectively.

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because clients offered a higher price, clients insisted on condom-free sex, and condoms as evidence of sex worker status. For partners, the reasons were familiarity, condom use being dependent on partner's decision, and condom use as evidence of sex worker status. There was no apparent relationship between HIV knowledge, time in sex work and safe sex practices.
Conclusion: Recommendations are made to improve the rate of condom use among FSW. These include establishing telephone help lines, establishing a FSW union, and designing HIV prevention programs for men.

P-B7 Perception and Misconception on HIV/AIDS and STIs among the Law instructors of the Police Training Centers in Bangladesh

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The South Asia Regional Office of International Organization for Migration (IOM) has taken an initiative on baseline information on the perception and misconception of police force on STI and HIV/AIDS in Bangladesh. Information on perception and misconceptions was gathered by pre-testing the police training instructors before starting the basic training on HIV and AIDS as a part of training need assessment. The training was arranged formally by seeking permission through the relevant ministry, Ministry of Home Affairs (MOHA), and the Inspector General of Police (IGP). Participants were from different Police Training Centers (PTCs) in the country. Different levels of police were addressed, as, commandant / commandant-In-charge, senior staff, drill and law Instructors, In-service trainees, cadet trainees (male), cadet trainees (female).
It was found that most of the police instructors (94%) think that they can be part of the HIV Prevention Program in the country. Most of them were found to have a positive perception by identifying themselves as vulnerable to STI and HIV/AIDS (92%). Only few knew that they can participate in HIV/AIDS prevention by protecting themselves and their families by understanding STI and HIV/AIDS (32%). Some found to have perception that they can prevent HIV/AIDS by using mosquito nets (30%) as they identified mosquito as one of the modes of transmission.
Some of the perceptions of the police instructors were also identified. As, condom promotion should not be encouraged (90%), frequent raid on sex workers place and IDU den should be done (30%). Nearly half of the participants thought that religious belief is sufficient for prevention of HIV and AIDS (45%) and MSM and transgender can be convicted under law (95%) of police penal code 290, Perverted sexual Act 377.

P-B8 Transferable Episomes of β-lactamase and Antibiogram as Epidemiological Markers of Gonorrhea

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Background: In Thailand, a high tendency of drug resistance and mobility of resistance genome have generally been found in many microbial populations including *Neisseria gonorrhoeae* isolates.
Methods: Herein, 235 isolates of *N. gonorrhoeae* from patients attending at Bangrak hospital, 68 isolates obtained in January-March 2000, 76 isolates obtained in January-March 2002, and 91 isolates obtained in October-December 2002, were tested.
Results: All isolates were susceptible to ceftriaxone while 71.1% and 76.6% were resistant to penicillin and quinolone, respectively. The percentage of penicillinase producing *N. gonorrhoeae* (PPNG) isolates increased from 48.5% in January-March 2000 to 84.6% in October-December 2002; however, high MIC (≥16 mg/L) was not different (34%). All 167 PPNG isolates except one could be identified β-lactamase producing plasmids by PCR and confirmed by *Hin*fI restriction enzyme digestion. The Asia plasmid was found in 24 isolates while 101 isolates contained the Africa plasmid and 41 isolates contained the Toronto plasmid. These epidemic plasmids were reported in each year with different percentages. The Africa type was markedly increased from 24.2% in January-March

2000 to 75.3% in October-December 2002 whereas the Asia and the Toronto type were highly decreased from 27.3% and 48.5% in January-March 2000 to 3.9% and 19.5% in October-December 2002. The Africa plasmid carrying isolates showed lower penicillin MIC comparing to the Asia and the Toronto plasmid carrying isolates. Furthermore, the Asia, the Africa, and the Toronto type plasmids could be transferred and continuously expressed in *Escherichia coli.*

Conclusion: These results indicate that gonococcal β-lactamase producing plasmids could be used as a marker for epidemiologically benefit along with the susceptibility pattern. Moreover, such a mobility of these episomes infers the high tendency of drug resistance development among infectious microorganism in the region.

P-B9 Acceptability of a penile wipe to promote better male genital hygiene in STI clinic attenders in Durban

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Background: Poor genital hygiene may be a risk factor for HIV infection. This study was done to determine whether a simple penile wipe is acceptable to clean both the prepuce and subpreputial space in male STI clinic attenders.

Methods: 650 male STI clinic attenders in Durban were enrolled into a study to investigate the relationship between male genital hygiene and HIV and asked to return after 14 days after treatment for STIs. Subjects were interviewed using a standard questionnaire about various aspects of genital hygiene. All men that returned were asked to apply a nappy wipe (Johnson's) to the prepuce in those circumcised or, in uncircumcised men, to the subpreputial space and prepuce by retracting the foreskin.

Results: The wipe was evaluated in 457 subjects that returned. The majority of the men (92%) were uncircumcised and 57 % were HIV positive. Washing before sex was reported by 6% and after sex by 30%. Patients' perception of their own personal genital hygiene was, very good (21%), quite good (54%), 50:50 (20%) and worse than 50:50 (5%) The wipe was acceptable to 99.8% of the men. Fifteen (3%) reported side effects of irritation (7), bleeding (1) and other (7). After use of the wipe, the prepuce was invariably slightly moist.

Conclusions: The wipe was acceptable to almost all subjects. The moist appearance of the prepuce after the application of the wipe would indicate that, if penile wetness is a factor in facilitating HIV transmission in uncircumcised men, the wipe should not be used either immediately before or after sex when exposure to HIV might be possible.

P-B10 High standard of penile hygiene in male STI clinic attenders in London

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Background: Poor genital hygiene may be a risk factor for HIV but there is little data relating to its assessment. This study was done to determine the standard of penile hygiene in men attending a London STI clinic.

Methods: 490 men attending Ealing Hospital GU clinic with a new problem were enrolled. Sociodemographic information was collected and standard STI tests performed. Genital examination was undertaken by two observers and the degree of subpreputial wetness classified as dry, wet, or very wet was assessed.

Results: The ethnic origins of the men were, Caucasian 249, Asian 111, Black 104 and Other 26. Overall, 359 (73%) were uncircumcised and 48 were homosexual. Six men with non-retractile foreskins were excluded from the analysis. Penile wetness of any degree was observed in 35(7%) of the men including four with profuse urethral discharge. Penile wetness was observed in 34 (9.5%) of