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ชื่อโครงการ (ไทย) การสร้างชุดทดสอบการดื้อยาริแฟมพิน
ของเชื้อวัณโรคด้วย PCR - reverse line blot
hybridization

(อังกฤษ) Development of test kit for detection of
rifampin resistance in *Mycobacterium
tuberculosis* by PCR - reverse line blot
hybridization

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บทคัดย่อ

การสร้างชุดทดสอบการดื้อยาริแฟมพินของเชื้อวัณโรคด้วย PCR - reverse line blot hybridization

วิธีการทดสอบทางพันธุศาสตร์โดยอาศัยเทคนิค PCR-reversed line blot hybridization ถูกพัฒนาขึ้นเพื่อตรวจหาเชื้อวัณโรคและการดื้อยาริแฟมพิน เมื่อใช้ปฏิกิริยา duplex PCR เพื่อเพิ่มปริมาณดีเอ็นเอเป้าหมายที่ IS6110 และ *rpoB* gene พบว่าความไวของการตรวจคือ 10 และ 100 fg ของดีเอ็นเอของ *M. tuberculosis* H37Rv ตามลำดับ เนื่องจาก duplex PCR มีความจำเพาะต่อเชื้อวัณโรคจึงสามารถนำมาใช้ทดสอบกับสิ่งส่งตรวจได้โดยตรง ทำการออกแบบ oligonucleotide probes เพื่อตรวจหา wild-type และ mutant genotypes ของ *rpoB* gene และนำ probes เหล่านี้พร้อมทั้ง probe จำเพาะของเชื้อวัณโรค ครึ่งเป็นแถวขนานกันบนแผ่นกระดาษกรองเพื่อนำมาทำไฮบริดเซชันกับ PCR product ที่ได้จากเชื้อและเสมหะของผู้ป่วย การทดสอบที่พัฒนาขึ้นนี้สามารถตรวจ clinical isolates ที่รู้ลำดับเบสของ *rpoB* gene แล้วได้อย่างถูกต้อง และตรวจพบเชื้อวัณโรค 98.75%(79/80) ในเสมหะของผู้ป่วย เมื่อนำ amplified products มาทดสอบด้วย line blot hybridization พบว่าสามารถตรวจพบเชื้อวัณโรคที่ดื้อยาริแฟมพิน 85.71% (24/28) ในเสมหะ และพบเชื้อวัณโรคที่ไวรับต่อยาได้ 100% ค่า clinical sensitivity และ specificity ของการทดสอบคือ 85.71% และ 100% ตามลำดับ เมื่อพิจารณาถึงเวลาที่ใช้ทดสอบ ค่าใช้จ่ายและอายุการใช้งานของแผ่นทดสอบแล้วจะเห็นว่า duplex PCR-reverse line blot hybridization มีศักยภาพที่จะใช้เป็นชุดทดสอบเพื่อตรวจหาการดื้อยาริแฟมพินในห้องปฏิบัติการทางคลินิก

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คำสำคัญ : เชื้อวัณโรค / วัณโรค / การดื้อยาริแฟมพิน / PCR- reverse line blot hybridization / IS6110 / *rpoB* gene

Abstract

Development of test kit for detection of rifampin resistance in *Mycobacterium tuberculosis* by PCR-reverse line blot hybridization

A genetic test by PCR-reverse line blot hybridization was developed for rapid detection of *Mycobacterium tuberculosis* and rifampin resistance simultaneously. Duplex PCR targeting IS 6110 and *rpoB* gene was employed to detect *M. tuberculosis* and the *rpoB* fragment with rifampin resistance hot spot region. The analytical sensitivity of duplex PCR for IS 6110 and *rpoB* gene was found to be 10 and 100fg of *M.tuberculosis* H37Rv DNA, respectively. Since duplex PCR was specific to *M. tuberculosis* complex, it was thus able to be applied directly in clinical specimens. Oligonucleotide probes were designed to detect wild –type and mutant genotypes of *rpoB* gene. These probes together with an *M. tuberculosis* specific probe were blotted in parallel lines in a membrane strip and used for hybridization with PCR product amplified from clinical isolates and sputum specimens. The developed test was able to detect *M .tuberculosis* clinical isolates with known *rpoB* gene sequences and the presence of *M. tuberculosis* in 98.75% (79/80) of sputum specimens. When the amplified products were subjected to reverse line blot hybridization, the test detected 85.71% (24/28) of rifampin-resistant and all rifampin-sensitive *M. tuberculosis* in sputum specimens. The clinical sensitivity and specificity of the test for rifampin resistance was 85.7% and 100%, respectively. Considering the turnaround time, cost and shelf life of the test , duplex PCR- reverse line blot hybridization offers a potential of the test kit for detection of frequent mutations leading to rifampin resistance in clinical laboratories.

Keywords: *Mycobacterium tuberculosis* / Tuberculosis / rifampin resistance / PCR-reverse line blot hybridization / IS 6110 / *rpoB* gene

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ABBREVIATIONS

A	adenine
AIDS	acquired immunodeficiency syndrome
bp	base pair
C	cytosine
CO ₂	carbon dioxide
°C	degree celsius
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
dGTP	deoxyguanosine 5'-triphosphate
dTTP	deoxythymidine 5'-triphosphate
DDW	double distilled water
DNA	deoxynucleic acid
dNTPs	deoxynucleotide - tri - phosphate
dPCR	duplex PCR
DW	distilled water
EDTA	ethylenediamine tetra-acetic acid
et al.	et alii
g	gram
G	guanine
HCl	hydrochloric acid
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
hr	hour
i.e.	in example
M.	Mycobacterium
M	molar
mg	milligram
MgCl ₂	magnesium chloride
min	minute (s)
ml	milliliter
mM	millimolar

NaCl	sodium chloride
NaOH	sodium hydroxide
NTM	non-tuberculous mycobacteria
PCR	polymerase chain reaction
pmol	picomol
RNA	ribonucleic acid
RFLP	restriction fragment length polymorphism
RLB	reverse line blot hybridization
rRNA	ribosomal ribonucleic acid
16S rRNA	16S ribosomal ribonucleic acid
16S rDNA	16S ribosomal deoxynucleic acid
s	second
T	thymine
TB	tuberculosis
TBE	tris-borate buffer
Taq	<i>Thermus aquaticus</i>
Tris	Tris - (Hydroxymethyl) - aminoethane
U	unit
µg	microgram
µl	microliter
µm	micromole
UV	ultraviolet
V	voltage

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INTRODUCTION

Tuberculosis (TB) remains to be the world's major public health problem. Overall, one-third of the world's population is currently infected with *Mycobacterium tuberculosis*. There was an estimation of 9.2 million new cases and 1.7 million deaths from TB in 2006 (1). The situation of TB was more severe with the development of drug resistant *M. tuberculosis* which commonly arises through the selection of mutated strains by inadequate treatment. Multidrug - resistant TB (MDR-TB) is a form of drug-resistant TB due to *M. tuberculosis* resistant to at least isoniazid and rifampin, the most powerful anti-TB drugs. There was an estimated 0.5 million cases of MDR-TB in 2006. Treatment of MDR-TB requires extensive chemotherapy with second-line drugs which have more toxicity for up to two years while drug-susceptible TB can be cured within six months with first-line drugs (1). MDR-TB cases should be detected and treated to ensure effective therapy and reduce transmission and spread of the disease (2, 3)

Conventional drug susceptibility testing with primary specimens has a long turnaround time of several weeks to months, which delays diagnosis of MDR-TB cases. Although several automated culture systems such as MB/BacT (Organon-Teknika), MGIT 960, BACTEC 460TB and BACTEC 9000 MB (Becton Dickinson) have been developed that allow a more rapid mycobacterial culture, it can take 8 to 18 days for culture (4,5,6) and subsequent assessment of drug susceptibility testing for 7 to 15 days (7,8,9). It is, therefore, needed to develop a simple, reliable and cost-affordable method to detect MDR-TB cases as soon as possible before the result of culture-based method can be obtained.

Rifampin is a key component for the effective short-course chemotherapy and rifampin - resistant *M. tuberculosis* strains are usually resistant to isoniazid (10,11). Rifampin resistance could then be a marker for MDR-TB. Rifampin resistance in *M. tuberculosis* is caused by a number of mutations in the *rpoB* gene encoding the β -subunit of RNA polymerase B. About 95% of these mutations occurred within a 81-bp hotspot region, the so-called rifampin resistance determining region (RRDR), encoding 27 amino acids and corresponding to codons 507-533 in the center of the 3,516-bp *rpoB* gene (12). The *rpoB* gene mutation is, therefore, the target for development of a rapid test for detection of MDR-TB.

Several rapid methods developed to detect *rpoB* gene mutation involved the amplification of *rpoB* gene by polymerase chain reaction (PCR) and the analysis of the PCR product by different methodology such as direct DNA sequencing (13,14,15), single-strand conformation polymorphism (11,13) , heteroduplex mobility assay (16) , the line probe assay (17,18,19), the GenoType MTBDR plus assay (20,21) and microarray analysis (22, 23). In addition to conventional PCR, real-time PCR was also applied to the detection of *rpoB* gene mutation (24, 25,26).

In clinical molecular diagnostics laboratory, the simplicity, turnaround time and cost of the test are major factors to be considered . The INNO-LiPA Rif. TB assay (Innogenetics, Ghent Belgium), a commercially available kit for the detection of *rpoB* mutations in rifampin-resistant *M.tuberculosis* , has been very successful in this application due to the simplicity and turnaround time (17,18,19). However, the high cost of this test limited its application worldwide especially in the developing countries. This test, based on reverse hybridization technique developed by Saiki et al. (27), allowed simultaneous detection of *M. tuberculosis* and identification of *rpoB* gene mutation by the hybridization patterns with probe lines on the membrane.

The aim of this study was to develop a rapid test to detect *M. tuberculosis* and *rpoB* gene mutation (s) in sputum specimen by using PCR followed by reverse hybridization with multiple probes fixed in parallel lines on a membrane (line blot). The validity of the test was evaluated by comparing the results with those obtained by MGIT 960 assay and DNA sequencing.

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MATERIALS AND METHODS

Bacterial strains. Eighteen reference strains of mycobacteria and 9 clinical isolates of nonmycobacteria (Table 1) together with 8 clinical isolates of *Mycobacterium tuberculosis* (Table 2) were used in this study. The reference strains were gift from Dr. Chiyoji Abe, Research institute of Tuberculosis, Japan Anti-Tuberculosis Association, Japan. Clinical isolates of nonmycobacteria were obtained from diagnostic laboratory of Department of Microbiology, Faculty of Medicine, Chulalongkorn University. Clinical isolates of *M. tuberculosis* comprised of 2 rifampin- sensitive and 6 rifampin-resistant strains for which the nucleotide sequence in the *rpoB* region was known (Table 2). These *M. tuberculosis* clinical isolates were analyzed for rifampin susceptibility by the proportion method with Lowenstein-Jensen egg slants and DNA sequencing of the amplified fragment of *rpo B* gene (28)

All mycobacterial strains were cultured on Ogawa medium at 37°C, except *M. marinum* at 30°C. *Escherichia coli*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Rhodococcus equi* were cultured on blood agar. *Nocardia asteroides*, and *Nocardia brasiliensis* were cultured on Sabouraud's dextrose agar at 25°C.

Preparation of chromosomal DNA. Purified chromosomal DNAs from mycobacterial reference strains as listed in Table1 were prepared by lysozyme-proteinase K-sodium dodecyl sulfate (SDS) procedure (29) with modification. DNAs prepared by this method were used to determine the sensitivity and specificity of duplex PCR. Briefly, culture on solid medium was suspended in 10 mM Tris-HCL, 1 mM EDTA (TE buffer) and subsequently heated at 100 °C for 10 min in a heating box to inactivate cells. Lysozyme was added to a final concentration of 1 mg/ml and the mixture was incubated at 37°C for 90 min. Proteinase K and SDS were added to final concentration of 0.1 mg/ml and 1%, respectively and the mixture was incubated overnight at 55°C. DNA was phenol extracted three times and ethanol precipitated. The DNA was dried in a heating box at 37°C for 1 h , dissolved in TE buffer and stored at -20°C until needed. The sample was checked for purity and determined for the amount of DNA by measuring the optical density at wavelengths of 260 nm and 280 nm.

Table 1. Mycobacteria and nonmycobacteria used to determine the specificity of duplex PCR-reverse line blot hybridization (RLB).

Microorganisms	duplex PCR		Pattern of RLB	
	<i>IS6110</i> (249 bp)	<i>rpo B</i> (157 bp)	Pt3	<i>rpoB</i> probes
Mycobacteria				
<i>M. tuberculosis</i> H37Rv KK 11-20	+	+	+	WT
<i>M. bovis</i> BCG KK 12-02	+	+	+	WT
<i>M. avium</i> ATCC 25291	-	+	-	NA
<i>M. intracellulare</i> ATCC 13950	-	+	-	NA
<i>M. abscessus</i> ATCC 19977	-	+	-	NA
<i>M. asiaticum</i> ATCC 25276	-	+	-	NA
<i>M. flavescens</i> JATA 67-01	-	+	-	NA
<i>M. fortuitum</i> ATCC 6841	-	+	-	NA
<i>M. goodii</i> ATCC 14470	-	+	-	NA
<i>M. kansasii</i> ATCC 12478	-	+	-	NA
<i>M. marinum</i> ATCC 927	-	+	-	NA
<i>M. nonchromogenicum</i> ATCC 19530	-	+	-	NA
<i>M. scrofulaceum</i> ATCC 19981	-	+	-	NA
<i>M. simiae</i> ATCC 14275	-	+	-	NA
<i>M. szulgai</i> KK 32-01	-	+	-	NA
<i>M. terrae</i> ATCC 15755	-	+	-	NA
<i>M. vaccae</i> KK 66-01	-	+	-	NA
<i>M. xenopi</i> ATCC 19250	-	+	-	NA
Nonmycobacteria				
<i>Escherichia coli</i>	-	+	-	NA
<i>Haemophilus influenzae</i>	-	+	-	NA
<i>Klebsiella pneumoniae</i>	-	+	-	NA
<i>Nocardia asteroides</i>	-	+	-	NA
<i>Nocardia brasiliensis</i>	-	+	-	NA
<i>Pseudomonas aeruginosa</i>	-	+	-	NA
<i>Rhodococcus equi</i>	-	+	-	NA
<i>Staphylococcus aureus</i>	-	+	-	NA
<i>Streptococcus pneumoniae</i>	-	+	-	NA

WT, wild type

NA, not applicable

Table 2. Clinical isolates of *M. tuberculosis* with known *rpo B* gene mutation.

Isolates	Number
<i>M. tuberculosis</i>	
Rifampin-sensitive	2
Rifampin-resistant	
516 GAC (Asp) → GTC (Val)	1
526 CAC (His) → TAC (Tyr)	1
526 CAC (His) → CGC (Arg)	1
526 CAC (His) → GAC (Asp)	1
531 TCG (Ser) → TTG (Leu)	1
531 TCG (Ser) → TGG (Trp)	1

Preparation of bacterial lysates. All clinical isolates were treated by the method described by Hall *et.al.*(30) as follow. Bacteria from one large colony or several small colonies were resuspended in 0.5 ml of alkaline wash solution composed of 0.05 M sodium citrate and 0.5 M NaOH by vortex and allowed to stand for 5 min. After centrifugation at 12,000 rpm for 5 min, the cell pellet was washed in 0.5 ml of 0.5 M Tris-HCl (pH 8.0) and resuspended in 100 µl of TE buffer. The suspension was then boiled at 100°C for 10 min, centrifuged at 12,000 rpm for 5 min to precipitate cellular debris. The supernatant was transferred to a sterile eppendorf tube and stored at -20 °C until needed.

Clinical specimens and specimen processing. Between October 2005 and December 2007, 1813 sputum specimens were received for the mycobacterial culture at the diagnostic laboratory of Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. The sputum specimens were first decontaminated with NaOH-sodium citrate-N-acetyl-L-cysteine method (31) and concentrated by centrifugation after decontamination. The concentrated specimens were examined microscopically after Kinyoun acid-fast staining, aliquotted 500µl into eppendorf tubes and inoculated onto Ogawa medium and MGIT 960 (Becton Dickinson, Sparks, Md.). The aliquots were stored at -70°C until analysis.

Identification of mycobacterial culture from sputum specimens.

Mycobacterial culture was identified by growth rate, pigmentation with and without exposure to light and biochemical reaction tests. For rapid identification, mycobacterial colonies were tested with AccuProbes (Accuprobe; Gen-Probe, Inc, San Diego, CA). The selection of appropriate probe depends on growth rate, pigmentation and colony appearance. In case that *M. tuberculosis* was identified, the clinical isolate was subjected to drug susceptibility testing upon the request of physician.

Drug susceptibility testing (DST) of *M. tuberculosis* . DST with isoniazid (INH), rifampin (RMP), ethambutol (EMB) and streptomycin (SM) was performed by the BACTEC MGIT 960 method (Becton Dickinson, Sparks, MD). Tests were performed with the standard concentration of 0.1 µg/ml INH, 1.0 µg/ml RMP, 3.5 µg/ml EMB and 0.8 µg/ml SM.

DNA extraction from sputum specimens. Based on drug susceptibility results, 80 specimens were randomly selected from the frozen aliquotted specimens for testing by PCR- reverse line blot hybridization. These specimens were thawed, centrifuged at 13,000xg for 15 min and subjected to DNA extraction by using QIAamp®DNA Mini kit (Qiagen Corporation, Germany) according to the manufacturer's directions. In brief, 200 µl of sample was added with 20 µl of proteinase K, 200 µl buffer AL and mixed by vortex for 15 s. After lysis for 10 min at 56°C to reach DNA yield, 200 µl absolute ethanol was added and mixed by vortex for 15 s. To remove drop from the inside of the lid, the tubes was spinned at 8,000 rpm for 1 min . The mixture was transferred to QIAamp spin column and centrifuged at 8,000 rpm for 1 min. QIAamp spin column was then washed with 500 µl buffer AW1 and 500 µl buffer AW2 followed by the spin at 8,000 rpm for 1 min and at 14,000 rpm for 3 min, respectively. After centrifugation the QIAamp spin column was placed in a clean 1.5 ml microcentrifuge tube and 200 µl buffer AE were added and incubated at room temperature for 1 min. After incubation, the suspension was centrifuged at 8,000 rpm for 1 min and the DNA containing eluted buffer was stored at -20°C, ready for the PCR analysis.

PCR for IS 6110 and *rpoB* gene. A duplex PCR with primers which detect IS 6110 of *Mycobacterium tuberculosis* complex (32) and the *rpoB* gene (13) was performed. The duplex PCR primers were Pt 18 (5'- GAACCGTGAGGGCATCGA GG-3'), 5'- biotinylated INS-2 (5'-GCGTAGGCGTCGGTGACAAA -3'), TR9 (5'-TCGCCGCGATCAAGGAGT-3') and 5'-biotinylated TR8 (5'-TGCACGTCGC GGACCTCCA - 3'). A product of 249 bp, amplified by Pt 18 and INS-2 was indicative of *M.tuberculosis* and smaller fragments of 157 bp amplified by TR9 and TR8 was positive signal for *rpoB* gene.

Amplification was performed in 50 μ l mixture containing 1x buffer, 2 mM $MgCl_2$, 200 μ M deoxynucleoside triphosphates (dNTPs; dATP, dCTP, dGTP, and dTTP), 1.25 U of *Taq* polymerase and primers (25 pmoles each of TR9 and TR8 , and 12.5 pmoles each of Pt18 and INS-2). The reaction was performed in 0.2 ml eppendorf tube with target DNA on a thermal cyclers (GeneAmp PCR System 9700, PE Applied Biosystems, USA). The PCR cycling parameters were 95 °C for 6 min, followed by 40 cycles of denaturation at 94°C for 1 min, primer annealing at 62°C for 1 min, extension at 72°C for 1 min, and one cycle of 72 °C for 10 min .

Sample of 5 μ l was used for PCR in a total volume of 50 μ l. In case of clinical sample, one part of treated specimen was spiked with 100 pg of *M. tuberculosis* DNA for testing of the amplification inhibitor in the sample. The sample was scored as negative if the test sample was negative and the spiked sample was positive. If both test and spiked sample were negative, then there was inhibitor in the sample.

Detection of amplification product. Five microliters of PCR product were mixed with 2 μ l of gel loading buffer (20% ficoll, 0.05% bromophenol blue), analyzed by electrophoresis on 2.0% agarose gel, consisted of 0.5 μ g/ml ethidium bromide, in 1X Tris-borate-EDTA (1XTBE) buffer (pH8.0). The electrophoresis was carried out at 80 volts for 40 min. Gel was visualized with UV fluorescence and the result was photographed by using the Bio-Rad Gel Doc system (Bio-Rad Laboratories, Hercules, CA).

Analytical sensitivity and specificity of duplex PCR. In order to determine the analytical sensitivity, purified DNA of *M.tuberculosis* H37Rv was diluted to a concentration of 2 ng/ μ l and then further 10x diluted in TE buffer to provide samples

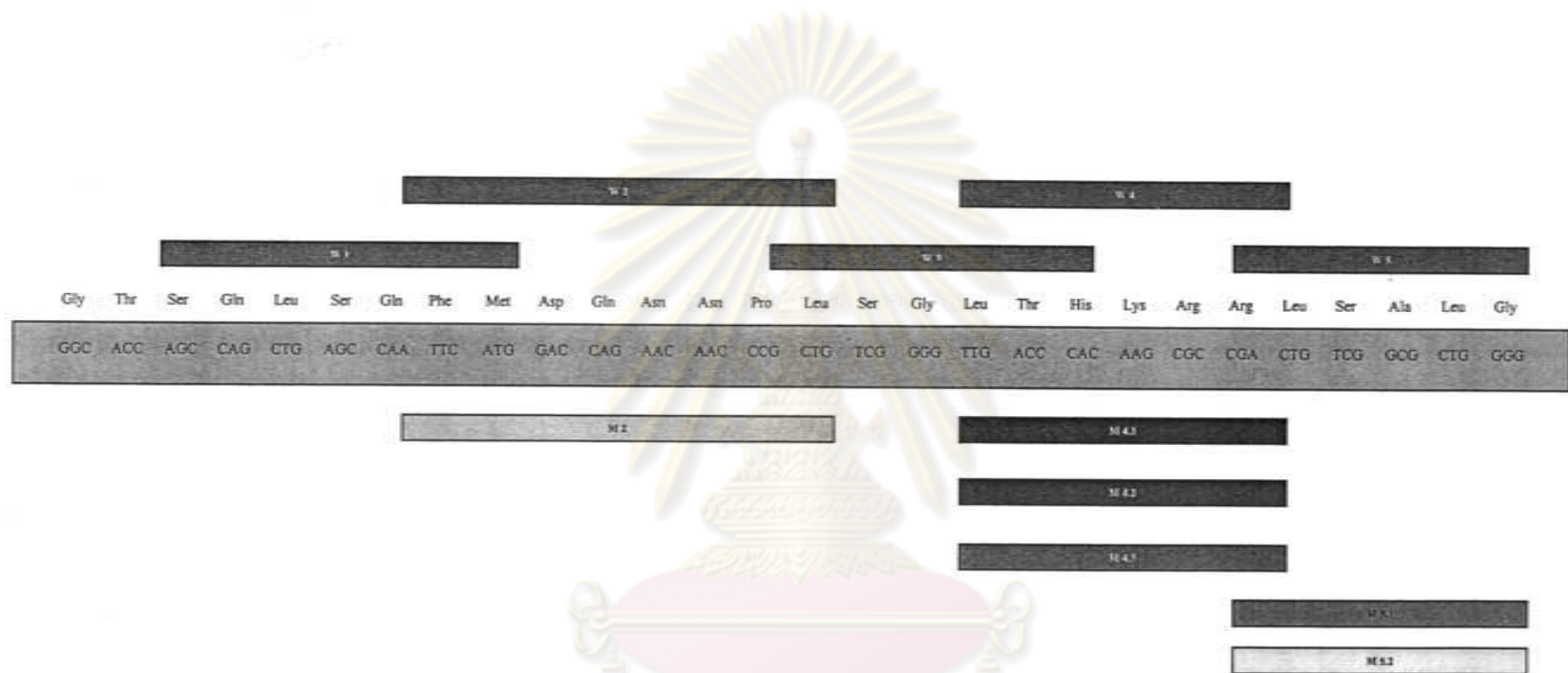
with concentration ranging from 200 pg to 0.2 fg of DNA per μl . Five microliters of these samples were amplified by duplex PCR. The amplified products (5 μl) were electrophoresed in 2% agarose gel containing ethidium bromide and visualized by UV fluorescence. The analytical specificity of duplex PCR was determined by using 10 ng of DNAs from microorganisms listed in Table 1. For negative control, DDW was used as the template for duplex PCR

Reverse line blot hybridization. In reverse line blot hybridization, the biotinylated PCR products of IS 6110 and *rpoB* gene were hybridized to a set of oligonucleotide probes which were covalently bound to Biodyne C membrane by line blotting in a Miniblotter MN 45 (Immunitics, MA, USA) (33) .The membrane was then cut into strip of desirable size for testing. The hybridized PCR product was detected by incubation with streptavidin-alkaline phosphatase and a color substrate of nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP solution, Roche Diagnostics GmbH, Penzberg, Germany). Oligonucleotide probes were designed based on the sequences of *rpoB* gene mutation of *M. tuberculosis*. A total of 11 oligonucleotide probes to detect wild type and mutant genotypes were designed and the diagram of the probes was shown in Figure1. *M. tuberculosis* positive control was included by using Pt3, an oligonucleotide probe specific to *M. tuberculosis* complex (32). These oligonucleotides were 5' amino linked (Table 3) .). The wild type probes W1,W2,W3,W4 and W5 overlap the entire *rpoB* hotspot region and the 6 mutant probes covered the mutations most frequently observed. The location of the probes on the strip was illustrated on the left side of Figure 4. The presence of rifampin-susceptible *M. tuberculosis* was demonstrated by the positive signal of hybridization with all wild-type probes and negative signal with all mutant probes. The presence of rifampin-resistant isolate was indicated either by the loss of hybridization of relevant wild-type probe with or without the hybridization with the mutant probe.

Table 3. Nucleotide sequences of the amino-link oligonucleotide probes.

Sequence (5'→3')	Type	Oligo name	Position (codon)
GAA CGG CTG ATG ACC AAA CT	<i>M. tuberculosis</i> complex	Pt3	-
AGC CAG CTG AGC CAA TTC ATG	Wild	W1	512 (AGC)
ATT CAT GGA CCA GAA CAA CCCG	Wild	W2	516 (GAC)
GCT GTC GGG GTT GAC CCA	Wild	W3	522 (TCG)
TTG ACC CAC AAG CGC CGA C	Wild	W4	526 (CAC)
ACT GTC GGC GCT GGG G	Wild	W5	531 (TCG)
TTC ATG GTC CAG AAC AAC CC	Mutant	M2	516 (GTC)
TTG ACC TAC AAG CGC CGA C	Mutant	M4.1	526 (TAC)
TTG ACC GAC AAG CGC CG	Mutant	M4.2	526 (GAC)
TTG ACC CGC AAG CGC CGA	Mutant	M4.3	526 (CGC)
ACT GTT GGC GCT GGG GC	Mutant	M5.1	531 (TTG)
ACT GTG GGC GCT GGG GC	Mutant	M5.2	531 (TGG)
GGT TGC ACG CAT TTTCCC TTAA	Conjugated control	Conjugated Control 5' aminolink and 3' biotinylated	-

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Figure 1. Schematic representation of the probes within the rifampin resistance determining region (RRDR) of the *rpoB* gene.

The reverse line blot hybridization assay was performed as follows.

1) Preparation of the reverse line blot. The 0.8x 10 cm strips of Biodyne C membrane were prehybridized in 10 ml freshly prepared 16% (w/v) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) in demineralized water at room temperature for 15 min. The membranes were placed in plastic container and shaken with demineralized water for 2 min. After the membranes were activated, they were placed on support cushion in a clean dot blotter apparatus (Bethesda Research Laboratories, USA). Dilutes of the oligonucleotide solution in 100 μ l 0.5 M NaHCO₃, pH 8.4 (Table 3) were added to the membranes by using Miniblotter. The membranes were incubated in 200 ml freshly prepared 0.1 M NaOH for 10 min (maximum) at room temperature in a plastic container and rinsed with demineralized water. The membranes were washed in 50 ml 2xSSPE/0.1% SDS for 10 min at 50°C and finally washed in 50 ml 20 mM EDTA, pH 8.0 for 15 min at room temperature

2) Reverse line blot hybridization. Twenty microliters of the PCR product were added to 1,500 μ l 2xSSPE/0.1% SDS, and heated for 10 min at 100°C and cool on ice immediately. The membrane was washed for 5 min at 50°C in 50 ml of 2xSSPE/0.1% SDS. The membrane was then placed into 15-ml hybridization tube and the tube filled with 1,500 μ l of diluted PCR product. Hybridization was performed for 60 min at 50°C in a hybridization oven (Thermo Hybaid, Middlesex, UK). After hybridization, the membrane was washed twice in 50 ml 2xSSPE /0.5% SDS for 10 min at 61°C.

3) Detection of hybridization signals. The membrane with the hybridized PCR product was placed in a plastic container and allowed to cool down before performing the color detection of hybridized PCR products as follows. Ten membranes were incubated with 20 ml blocking solution for 30 minutes. The membranes were then washed 3 times for 10 minutes each with TBS (0.05 M Tris-HCl, 0.15 M NaCl, pH 7.5). Solution of 5 μ l streptavidin-alkaline phosphatase conjugate (1000 U/ml) was added to 10 ml TBS, 0.1% Tween 20 (w/v) and the membranes were incubated in this solution for 1 hour. After washing 3 times for 10 minutes each with 50 ml TBS, the membranes were immersed in the NBT/BCIP staining solution prepared as the instruction of the manufacturer. When the development of the blue color was observed, the membranes were rinsed two times

with distilled water and dried on paper towels. The membranes were stored under light protection for documentation.

Sequencing of the amplified *rpoB* gene. An ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystem) was used for the sequencing of the PCR product. Sequencing was performed on 30-90 ng of purified PCR product using QIAquick PCR Purification Kit; Qiagen Corporation, Germany. The sequencing reaction required 4 ml of Premix, 3.2 pmol of sequencing primer TR9, 150 ng of PCR product template in a total volume of 10 ul. The sequencing reaction was performed using Perkin Elmer GeneAmp PCR system 9600 with cycling parameters were 25 cycles of 96° C for 30 sec, 55° C for 10 sec, 60° C for 4 min and hold 4° C until ready to purify. The products were purified with ethanol/sodium acetate precipitation before capillary electrophoresis was run for sequence analysis.



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RESULTS

Amplification of IS 6110 and *rpoB* gene by duplex PCR. The analytical sensitivity of duplex PCR was tested with serial 10-fold diluted purified DNAs from *M. tuberculosis* H37Rv. The PCR products were electrophoresed on 2% agarose gel containing ethidium bromide. From Figure 2, it was shown that a 249-bp fragment of IS 6110 and a 157-bp fragment of *rpoB* gene were amplified with the analytical sensitivity of 10 and 100 fg of DNA, respectively.

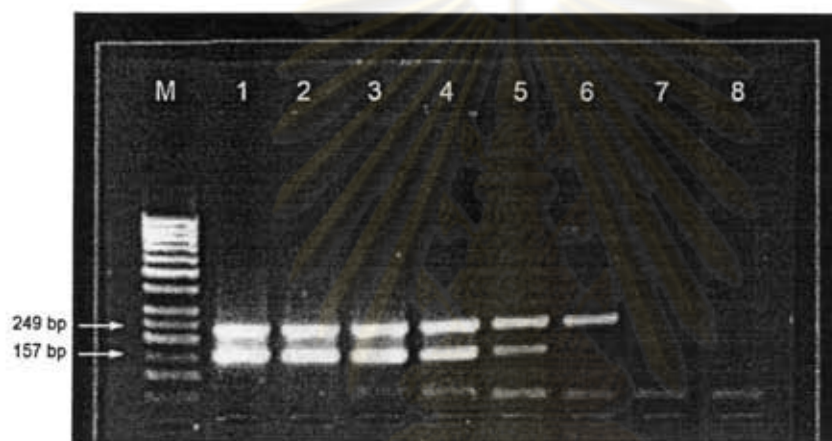


Figure2. Analytical sensitivity of duplex PCR for detection of *M. tuberculosis* H37Rv. Lanes: M, molecular weight marker; 1, *M. tuberculosis* DNA 1 ng ; 2, 100 pg ; 3, 10 pg; 4, 1 pg ; 5, 100 fg ; 6, 16 fg ; 7, 1 fg ; 8, negative control.

The specificity of duplex PCR was tested with 10 ng of purified chromosomal DNAs from mycobacteria and lysates of clinical isolates as listed in Tables1. No amplification of IS 6110 was found in non-tuberculous mycobacteria and the other tested microorganisms. Only *M. bovis* which belongs to *M. tuberculosis* complex gave a 249-bp fragment of IS 6110 amplification. A single band of approximate 157-bp of *rpoB* gene was found in all amplified DNAs from tested microorganisms (Table 1).

Results of duplex PCR and reverse line blot hybridization (dPCR-RLB) tested with DNAs of microorganisms. In order to evaluate dPCR-RLB for detection of rifampin- resistant *M. tuberculosis*, clinical isolates of *M. tuberculosis* with known *rpoB* mutations and wild type were tested first. Clinical isolates as listed in Table 2 were subjected to DNA extraction by alkaline wash and heat lysis method and the extracted DNAs were amplified by duplex PCR. Expected DNA bands of 249- and 157-bp were seen from all clinical isolates (Figure 3) . All hybridized with *M. tuberculosis* probe (Pt3) and each PCR product hybridized with its-specific probe (s). The product of wild type gave positive hybridization signal with all wild type probes and negative signal with all mutant probes (Figure 4, lanes 3 and 4). The product of each mutant gave positive signal with its mutant probe with the disappearance of the relevant wild type probe (Figure 4, lanes 5-9). However, the products of rifampin - resistant isolate which contains mutation at amino acid 531 (Ser →Leu) gave faint hybridization signals with the mutant probes of amino acid 526 (Probes M 4.1, M 4.2, M 4.3) and mutant probe of amino acid 531 Ser → Trp (probe M 5.2) as shown in Figure 3, lane 8.

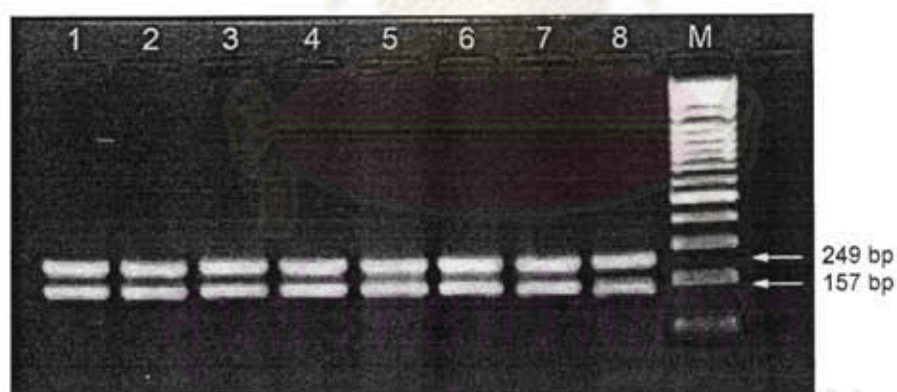


Figure 3. Amplification of mycobacterial DNA from clinical isolates with known *rpoB* gene sequence. Lanes: M, molecular weight marker ; 1&2, wild type; 3, *rpoB* 516 GAC(Asp)→ GTC (Val) ; 4, *rpoB* 526 CAC (His) →TAC(Tyr) ; 5, *rpoB* 526 CAC (His) →GAC(Asp) ; 6, *rpoB* 526 CAC (His) →CGC(Arg) ; 7, *rpoB* 531 TCG (Ser) →TTG(Leu) ; 8, *rpoB* 531 TCG (Ser) →TGG(Trp) .

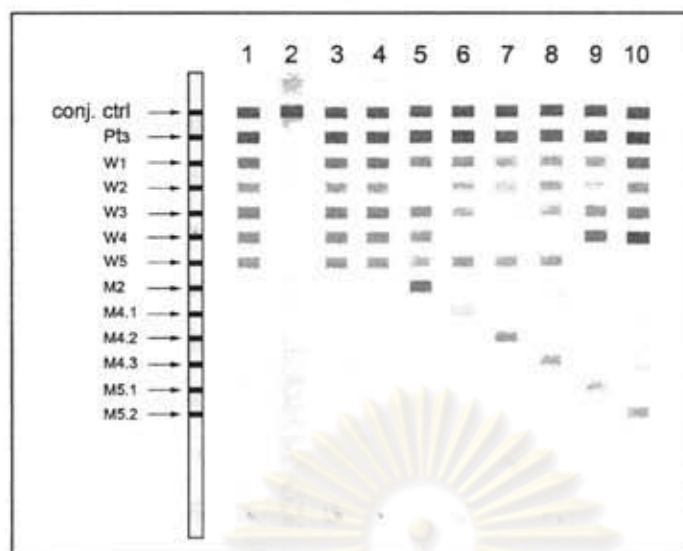


Figure 4. Hybridization patterns obtained with line blot hybridization. Strip design showing the positions of probes and the marker line is given on the left side. Patterns of the strips for wild- type and mutant *M. tuberculosis clinical* isolates are shown on the right. Lanes: 1, *M. tuberculosis* H37Rv; 2, negative control; 3&4, wild-type; 5, *rpoB* 516 GAC(Asp)→ GTC (Val) ; 6, *rpoB* 526 CAC (His) →TAC(Tyr) ; 7, *rpoB* 526 CAC (His) →GAC(Asp) ; 8, *rpoB* 526 CAC (His) →CGC(Arg) ; 9, *rpoB* 531 TCG (Ser) →TTG(Leu) ; 10, *rpoB* 531 TCG (Ser) →TGG(Trp) .

The line blot was also tested by using the microorganisms as listed in Table 1. Only amplified products of *M. tuberculosis* H37Rv and *M. bovis* hybridized with *M. tuberculosis* complex probe (Pt3) and all wild-type probes of *rpoB* gene. The amplified products of non-tuberculous mycobacteria and the other microorganisms did not hybridize with probe Pt3 and most of the probes in the line blots (Table 1).

Results of duplex PCR and reverse line blot hybridization (dPCR-RLB) tested in clinical specimens compared with results of MGIT method. During the period of study, 803 *M. tuberculosis* isolates were grown from sputum specimens and 234 were tested for drug susceptibility. Eighty specimens were selected from the frozen aliquots for testing by dPCR-RLB. These samples comprised of all available sputum specimens which grew rifampin-resistant *M. tuberculosis* (n=28) and 52 randomly chosen specimens which grew rifampin-sensitive *M. tuberculosis*. The extracted DNAs were amplified by duplex PCR and the products were subjected to

RLB. The dPCR-RLB produced interpretable result in 79 (98.75%) sputum samples. DNA bands of 249- and 157-bp were amplified (Figure 5) and the products hybridized to their relevant probes. (Figure 6). Rifampin susceptibility could not be determined in one sputum sample by dPCR-RLB. In this sample, only a faint DNA band of 249-bp showed up. This indicated the presence of *M. tuberculosis DNA* in the sample but the *rpoB* gene fragment was not amplified. RLB hybridization of this sample did not show positive signal with any probe (data not shown). Comparable results of rifampin susceptibility between MGIT 960 and dPCR-RLB were demonstrated in Table 4. dPCR-RLB correctly detected all 52 rifampin-sensitive *M. tuberculosis* in sputum specimens and 24 out of 28 (85.71%) rifampin-resistant *M. tuberculosis* in sputum specimens. The discrepant results were found in 3 (10.71%) samples which were interpreted to be resistant by MGIT 960 and susceptible by dPCR-RLB. The DNA sequences of amplified products obtained from both *M. tuberculosis* isolates and directly from sputum samples revealed the wild-type sequences in these three specimens.



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Figure 5. Amplification of DNAs extracted from sputum specimen by duplex PCR. Lanes: 1-10, sputum specimens ; 11, negative control ; 12, positive control.

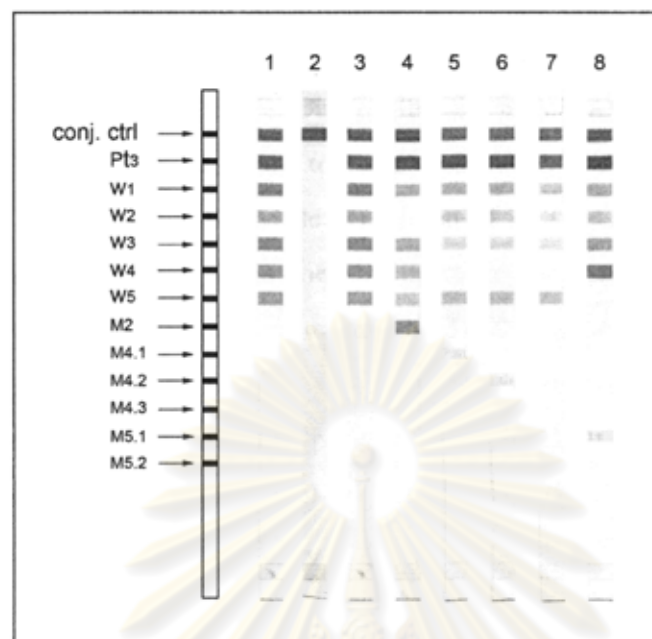


Figure 6. Representative result of hybridization patterns obtained with line blot hybridization assay with sputum specimens. Patterns of the strips for wild- type and mutant *M. tuberculosis clinical* isolates are shown on the right. Lanes: 1, *M. tuberculosis* H37Rv; 2, negative control; 3, wild-type; 4, *rpoB* 516 GAC(Asp)→ GTC (Val) ; 5, *rpoB* 526 CAC (His) →TAC(Tyr) ; 6, *rpoB* 526 CAC (His) →GAC(Asp) ; 7, *rpoB* 526 mutation; 8, *rpoB* 531 TCG (Ser) →TTG(Leu).

Table 4. Rifampin susceptibility of *M. tuberculosis* in sputum specimens determined by dPCR-RLB and MGIT

dPCR-RLB result	No. of <i>M. tuberculosis</i> isolates with the MGIT testing to be	
	Susceptible (n=52)	Resistant (n=28)
Susceptible	52	3
Resistant	-	24
Not determined ^a	-	1

^a RLB could not be interpreted due to no amplification

The mutations detected by RLB were confirmed in the resistant *M. tuberculosis* isolates from sputum samples by DNA sequencing. The result of antibiotic susceptibility, dPCR-RLB and DNA sequencing obtained from all 80 sputum specimens were shown in Table 5. Out of the 28 isolates which were rifampin-resistant, 25 (89.28%) were resistant to isoniazid and thus considered to be MDR isolates. The dPCR-RLB was able to detect 21 out of 25 (84.00%) MDR TB cases. *M. tuberculosis* culture isolate from the specimen that was negative by PCR was found to be MDR strain and had mutation at 531 Ser→ Leu (specimen no.10 in Table5).

The most common mutation genotype was 531 Ser→ Leu which was found in 13 of the 24 rifampin- resistant cases (54.16%) detected by dPCR-RLB. The frequency of mutation was demonstrated in Table 6.

Table 5. Comparison of results determined by MGIT, dPCR-RLB and DNA Sequencing to determine rifampin susceptibility of *M. tuberculosis* in sputum specimens.

Specimen no.	Code	Resistance to				Result from	
		RIF	INH	EMB	STR	PCR-RLB	DNA Sequencing
1	137/2	R	R	R	R	MUTANT5.1	531 TCG (Ser) → TTG (Leu)
2	65/3	R	R	S	S	MUTANT5.1	531 TCG (Ser) → TTG (Leu)
3	87/4	R	R	S	R	MUTANT4.3	526 CAC (His) → CGC (Arg)
4	153/4	R	R	S	R	MUTANT4.1	526 CAC (His) → TAC (Tyr)
5	31/7	R	R	S	R	MUTANT2	516 GAC (Asp) → GTC (Val)
6	37/9	R	R	S	R	MUTANT5.1	531 TCG (Ser) → TTG (Leu)
7	19/12	R	R	S	R	MUTANT2	516 GAC (Asp) → GTC (Val)
8	21/12	R	S	S	R	MUTANT4.1	526 CAC (His) → TAC (Tyr)
9	246/1	R	R	S	R	negative	531 TCG (Ser) → TTG (Leu) *
10	105/2	R	R	S	S	MUTANT4	526 CAC (His) → GTC (Val)
11	124/2	R	R	S	R	MUTANT2	516 GAC (Asp) → GTC (Val)
12	150/2	R	R	S	R	MUTANT4.1	526 CAC (His) → TAC (Tyr)

Specimen no.	Code	Resistance to				Result from	
		RIF	INH	EMB	STR	PCR-RLB	DNA Sequencing
13	186/2	R	S	S	S	MUTANT5.1	531 TCG (Ser) → TTG (Leu)
14	39/3	R	S	S	S	MUTANT4.2	526 CAC (His) → GAC (Asp)
15	140/3	R	R	S	R	WT	WT
16	165/3	R	R	S	S	MUTANT5.1	531 TCG (Ser) → TTG (Leu)
17	96/5	R	R	S	R	WT	WT
18	168/6	R	R	S	R	WT	WT
19	20/8	R	R	S	R	MUTANT5.1	531 TCG (Ser) → TTG (Leu)
20	91/9	R	R	S	S	MUTANT4	526 CAC (His) → CCC (Pro)
21	6/11	R	R	S	R	MUTANT5.1	531 TCG (Ser) → TTG (Leu)
22	94/11	R	R	S	S	MUTANT5.1	531 TCG (Ser) → TTG (Leu)
23	101/12	R	R	S	R	MUTANT5.1	531 TCG (Ser) → TTG (Leu)
24	18/6	R	R	S	R	MUTANT5.1	531 TCG (Ser) → TTG (Leu)
25	35/5	R	R	R	R	MUTANT4.1	526 CAC (His) → TAC (Tyr)
26	115/12	R	R	S	S	MUTANT5.1	531 TCG (Ser) → TTG (Leu)
27	106/1	R	R	S	R	MUTANT5.1	531 TCG (Ser) → TTG (Leu)
28	118/1	R	R	S	R	MUTANT5.1	531 TCG (Ser) → TTG (Leu)
29	66/1	S	S	S	S	WT	ND
30	19/3	S	S	S	S	WT	ND
31	64/3	S	S	S	S	WT	ND
32	103/3	S	R	S	S	WT	ND
33	143/3	S	R	S	R	WT	ND
34	160/3	S	S	S	S	WT	ND
35	163/3	S	R	S	S	WT	ND
36	117/3	S	S	S	S	WT	ND
37	89/5	S	R	R	S	WT	ND
38	93/5	S	S	S	S	WT	ND
39	116/5	S	S	S	R	WT	ND
40	133/5	S	S	S	S	WT	ND
41	134/5	S	R	S	S	WT	ND
42	149/5	S	S	S	R	WT	ND
43	20/6	S	S	S	S	WT	ND
44	61/6	S	S	S	S	WT	ND

Specimen no.	Code	Resistance to				Result from	
		RIF	INH	EMB	STR	PCR-RLB	DNA Sequencing
45	101/6	S	S	S	R	WT	ND
46	164/6	S	S	S	S	WT	ND
47	180/6	S	R	S	S	WT	ND
48	181/6	S	R	S	S	WT	ND
49	59/6	S	S	S	S	WT	ND
50	21/7	S	S	S	R	WT	ND
51	83/7	S	R	S	R	WT	ND
52	84/7	S	R	S	R	WT	ND
53	93/7	S	S	S	S	WT	ND
54	97/7	S	S	S	S	WT	ND
55	117/7	S	S	S	S	WT	ND
56	130/7	S	S	S	S	WT	ND
57	124/7	S	R	S	S	WT	ND
58	68/8	S	S	S	S	WT	ND
59	78/8	S	S	S	S	WT	ND
60	84/8	S	S	S	S	WT	ND
61	92/8	S	S	S	S	WT	ND
62	63/9	S	R	S	R	WT	ND
63	86/9	S	R	S	S	WT	ND
64	88/9	S	S	S	S	WT	ND
65	104/9	S	S	S	S	WT	ND
66	7/10	S	S	S	S	WT	ND
67	36/10	S	S	S	S	WT	ND
68	57/10	S	S	S	S	WT	ND
69	70/10	S	S	S	S	WT	ND
70	132/10	S	S	S	S	WT	ND
71	19/11	S	S	S	S	WT	ND
72	27/11	S	S	S	S	WT	ND
73	45/11	S	S	S	S	WT	ND
74	51/11	S	S	S	S	WT	ND
75	136/11	S	R	S	S	WT	ND
76	2/12	S	S	S	S	WT	ND
77	6/12	S	S	S	S	WT	ND

Specimen no.	Code	Resistance to				Result from	
		RIF	INH	EMB	STR	PCR-RLB	DNA Sequencing
78	24/12	S	S	S	S	WT	ND
79	42/12	S	S	S	S	WT	ND
80	113/12	S	R	S	S	WT	ND

* sequence from culture ; WT, wild type; ND, not determined

Table 6. Frequency of mutations in rifampin- resistant *M. tuberculosis* directly detected in sputum specimens.

RLB Pattern	Mutation	No. of sample (n=24)	DNA Sequencing
Mutant 2	516 Asp → Val	3	516 GAC (Asp) → GTC (Val)
Mutant 4.1	526 His → Tyr	4	526 CAC (His) → TAC (Tyr)
Mutant 4.2	526 His → Asp	1	526 CAC (His) → GAC (Asp)
Mutant 4.3	526 His → Arg	1	526 CAC (His) → CGC (Arg)
Mutant 4	526	1	526 CAC (His) → CCC (Pro)
Mutant 4	526	1	526 CAC (His) → GTC (Val)
Mutant 5	531 Ser → Leu	13	531 TCG (Ser) → TTG (Leu)

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DISCUSSION

Based on the knowledge of the molecular mechanism of rifampin resistance in *M. tuberculosis*, a number of genotypic assays have been developed for detection of mutant genotype. Most of the assays are practical in the research laboratory. PCR-reverse hybridization on a strip, using the commercial LiPA kit has proven to be robust, reproducible and easy to interpret (17, 18, 19). However, the high cost of this test, approximately 3,000 baht per strip in Thailand, prohibited its usage in most clinical laboratories. PCR-reverse hybridization assays have been developed as in-house assays for rapid detection of rifampin – resistance (33) and multidrug-resistance (34) in *M. tuberculosis* isolates. These assays have been successful in detection of drug resistance in bacterial culture but have not applied to the direct detection in clinical specimen.

In this study, duplex PCR - reverse hybridization was developed as assay for the rapid detection of *M.tuberculosis* and identification of *rpoB* gene mutation in clinical samples. In our assay, the duplex PCR protocol gave the sensitivity of detection of *M.tuberculosis* H37Rv of 10 and 100fg DNA for *IS 6110* and *rpoB* gene, which was equivalent to 2 and 20 cells, respectively. This was due to the 17 copy number of *IS 6110* and one copy number of *rpoB* gene in *M. tuberculosis* H37Rv genome. Duplex PCR also specifically detected *M. tuberculosis* complex with *IS 6110* amplification, thus was applicable directly in clinical specimens. Only the specimen that showed a 249- bp fragment were considered to contain *M. tuberculosis* and subjected to line blot hybridization. The sensitivity of detection of *M. tuberculosis* in sputum specimen by duplex PCR was found to be 98.75%. The only one sputum that was not positive by duplex PCR was AFB smear –negative. *M. tuberculosis* isolate from this specimen by culture method was found to be MDR strain and had mutation at 531 Ser→ Leu

For reverse line blot hybridization, the probes were able to detect all 52 wild-type genotype which were 100% concordant with the results obtained by MIGIT 960. Therefore, the clinical specificity of the test was 100%. The clinical sensitivity of the test was found to be 85.71% (24 out of 28) for detection of rifampin resistance and 84.00% (21 out of 25) for MDR cases. *M. tuberculosis* culture isolate from the specimen that was negative by dPCR was correctly identified by reverse line blot

hybridization to contain mutation at 531 Ser→ Leu. Three rifampin –resistant isolates contained no mutation within the hot spot region of *rpoB* gene. This may be due to the mutation outside the hot spot region or the existence of additional gene responsible for rifampin resistance (12,13,14, 16,18).

As with other genotype-based resistance prediction tests, a drawback in characterizing *rpoB* gene mutation is that no prediction can be made in the sample containing the non-mutated genotype(25) In case of the wild-type genotype was found, the result of culture-based drug susceptibility test should be confirmed although the result might be obtained in several weeks. This developed test was more useful in the rapid detection of rifampin resistance than rifampin susceptibility.

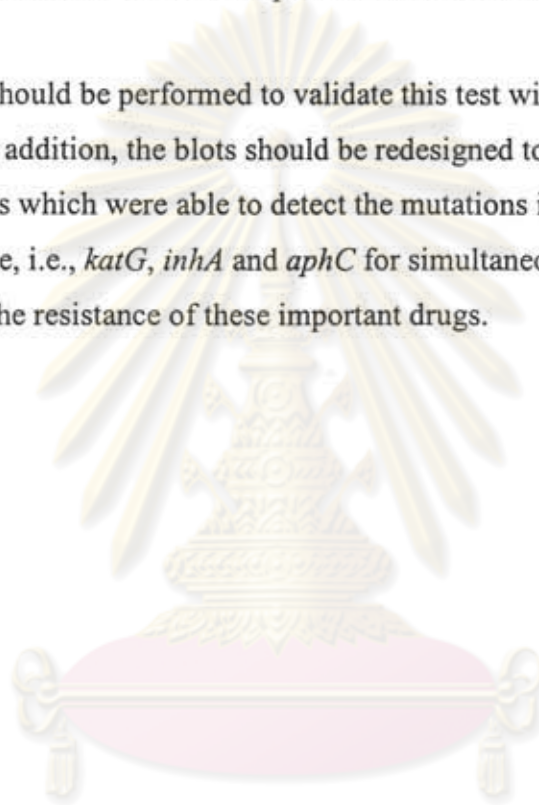
The developed multiplex PCR-reverse hybridization was a low-cost alternative to commercial kit for rapid detection *M. tuberculosis* complex and rifampin resistance. Results with duplex PCR assay can be obtained within 6 hours (extraction and reaction set up, 80 min; amplification, 4 hr by conventional PCR machines; and detection, 40 min) upon receipt of clinical specimens. The reverse line blot hybridization step takes approximately 4 hours. In practice, the turnaround time of duplex PCR-reverse hybridization is 2 to 3 days. The cost of the duplex PCR is approximately 320 baht per sample (excluding positive and negative control which cost 160 baht per run) and the cost of reverse line blot hybridization is approximately 320 baht per strip. In addition, the probe strip was found to be good for up to a year when sealed in plastic and kept at 4°C.

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CONCLUSION AND SUGGESTION

The duplex PCR-reverse line blot hybridization (dPCR-RLB) was developed for rapid detection of *Mycobacterium tuberculosis* and rifampin resistance. The assay was successful in testing with mycobacterial culture and sputum specimens . Considering the turnaround time, cost and shelf life of the test , dPCR-RLB offers a potential of the test kit for detection of frequent mutations leading to rifampin resistance.

Further study should be performed to validate this test with more number of clinical specimens. In addition, the blots should be redesigned to include oligonucleotide probes which were able to detect the mutations in genes responsible for isoniazid resistance, i.e., *katG*, *inhA* and *aphC* for simultaneous detection of mutations leading to the resistance of these important drugs.



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ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

ประวัติผู้วิจัย

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6. สาขาวิชาที่มีความชำนาญพิเศษ (แตกต่างจากวุฒิการศึกษา) ระบุสาขาวิชา
Molecular Bacteriology
7. ประสบการณ์ที่เกี่ยวข้องกับการบริหารงานวิจัยทั้งภายในและภายนอกประเทศ โดย
ระบุสถานภาพ ในการทำการวิจัยว่าเป็นผู้อำนวยการแผนงานวิจัย หัวหน้า
โครงการวิจัย หรือผู้ร่วมงานวิจัย
 - 7.1. หัวหน้าโครงการวิจัย : ชื่อโครงการวิจัย
 - การสร้างชุดทดสอบสำหรับตรวจสปีชีส์ของ *Mycobacterium* ด้วย reverse hybridization
 - การเปรียบเทียบวิธีตรวจหาความไวรับด้อยยา clarithromycin ของเชื้อ *Mycobacterium avium* complex
 - ความสัมพันธ์ระหว่างยีน *cagA*, *vacA*, *iceA* ของเชื้อเฮลิโคแบคเตอร์ ไพโลไร กับโรคแผล เปปติคในผู้ป่วยไทย
 - การศึกษาแบบสหสถาบันเพื่อศึกษาอินเตอร์ลิวคินในผู้ป่วยมะเร็งกระเพาะอาหารและแผลในกระเพาะอาหาร

- การจำแนกสปีชีส์ของเชื้อมัยโคแบคทีเรีย โดยการหาลำดับเบสของชิ้นส่วน 16S rDNA

- การพัฒนาเทคนิคทางชีวโมเลกุลเพื่อตรวจหาแบคทีเรียที่เพาะเลี้ยงยาก

7.2. งานวิจัยที่ทำเสร็จแล้ว

- 1) Vilaichone RK, Mahachai V, **Tumwasorn S**, Wu JY, Graham DY, Yamaoka Y. Molecular epidemiology and outcome of *Helicobacter pylori* infection in Thailand : a cultural cross roads. *Helicobacter* 2004; 9: 453-459
- 2) Jitsurong S, Chirathaworn C, Brown NF, Beacham IR, **Tumwasorn S**. Searching for virulence *Burkholderia pseudomallei* gene by immunoscreening the lamda ZAP III expressed genomic library. *Southeast Asian J Trop Med Public Health* 2003; 34: 810-821
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- 6) Vilaichone RK, Mahachai V, **Tumwasorn S**, Nanthapisud P, Wisedopas N, Kullavanijaya P. Duodenal *Helicobacter pylori* associated duodenal ulcer depend on gastric *Helicobacter pylori* status. *J Med Assoc Thai* 2002; Suppl 1: S97-102
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9) **Tumwasorn S, Nilgate S, Udomsantisuk N.** Amplification of P1 gene by polymerase chain reaction for detection of *Mycoplasma pneumoniae*. *J Med Assoc Thai* 2002; Suppl 1: S389-398

10) Hirunwiwatkul P, **Tumwasorn S, Chantranuwat C, Sirichai U. A** comparative studies of diagnostic tests of tuberculous lymphadenitis: polymerase chain reaction vs histopathology and clinical diagnosis. *J Med Assoc Thai* 2002; 85(3): 320-326

7.3. งานวิจัยที่กำลังทำ

- การสร้างชุดทดสอบสำหรับตรวจสปีชีส์ของ *Mycobacterium* ด้วย reverse hybridization

แหล่งทุน : ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ

สถานภาพ : ทำลุล่วงแล้ว 70%

- การเปรียบเทียบวิธีตรวจหาความไวรับต่อยา clarithromycin ของเชื้อ *Mycobacterium avium* complex

แหล่งทุน : เงินทุนวิจัยรัชดาภิเษกสมโภช คณะแพทยศาสตร์ จุฬาลงกรณ์

มหาวิทยาลัย และทุนสนับสนุนวิทยานิพนธ์และกลุ่มวิทยานิพนธ์ จุฬาลงกรณ์มหาวิทยาลัย

สถานภาพ : ทำลุล่วงแล้ว 80%

- ความสัมพันธ์ระหว่างยีน *cagA*, *vacA*, *iceA* ของ เชื้อเฮลิโคแบคเตอร์ ไพโลไร กับโรคแผล เปปติคในผู้ป่วยไทย

แหล่งทุน : เงินทุนวิจัยชีวโมเลกุล คณะแพทยศาสตร์ จุฬาลงกรณ์

มหาวิทยาลัย และทุนสนับสนุนวิทยานิพนธ์และกลุ่มวิทยานิพนธ์ จุฬาลงกรณ์มหาวิทยาลัย

สถานภาพ : ทำลุล่วงแล้ว 90%

จุฬาลงกรณ์มหาวิทยาลัย

ประวัติผู้วิจัย

1. ชื่อ-สกุล (ภาษาไทย) นางสาวอัจฉราพร สวัสดิ์พานิช
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วท.บ. (จุลชีววิทยา) ม.ขอนแก่น, 2538
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Diagnostic Molecular Bacteriology
7. ประสบการณ์ที่เกี่ยวข้องกับการบริหารงานวิจัยทั้งภายในและภายนอกประเทศ โดยระบุสถานภาพ ในการทำการวิจัยว่าเป็นผู้อำนวยการแผนงานวิจัย หัวหน้าโครงการวิจัย หรือผู้ร่วมงานวิจัย
 - 7.1. ผู้ร่วมโครงการวิจัย : ชื่อโครงการวิจัย
 - การสร้างชุดทดสอบสำหรับตรวจสปีชีส์ของ *Mycobacterium* ด้วย reverse hybridization
 - การเปรียบเทียบวิธีตรวจหาความไวรับต่อยา clarithromycin ของเชื้อ *Mycobacterium avium* complex
 - ความสัมพันธ์ระหว่างยีน *cag A*, *vac A*, *ice A* ของเชื้อ เฮลิโคแบคเตอร์ ไพโลไร กับ โรคแผล เปปติคในผู้ป่วยไทย
 - 7.2. งานวิจัยที่ทำเสร็จแล้ว
 - 1) Tumwasorn S, Leetranont S, Sawatpanich A, Vilaichone R, Mahachai V. Detection of clarithromycin resistance in *Helicobacter pylori* isolated from Thai patients. *Helicobacter* 2004; 9: 585 Abstract no: 13.06

- 2) **Sawatpanich A.** Polymerase chain reaction amplification of gene encoding P1 protein and 16S rRNA for detection of *Mycoplasma pneumoniae*.
Master's Thesis, Inter-Department of Medical Microbiology, Graduate School, Chulalongkorn University

7.4. งานวิจัยที่กำลังทำ

- การสร้างชุดทดสอบสำหรับตรวจสปีชีส์ของ *Mycobacterium* ด้วย reverse hybridization
แหล่งทุน : ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ
สถานภาพ : ทำลุล่วงแล้ว 70%
- การเปรียบเทียบวิธีตรวจหาความไวรับต่อยา clarithromycin ของเชื้อ *Mycobacterium avium* complex
แหล่งทุน : เงินทุนวิจัยรัชดาภิเษกสมโภช คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย และทุนสนับสนุนวิทยานิพนธ์และกลุ่มวิทยานิพนธ์ จุฬาลงกรณ์มหาวิทยาลัย
สถานภาพ : ทำลุล่วงแล้ว 80%
- ความสัมพันธ์ระหว่างยีน *cagA*, *va A*, *ice A* ของเชื้อเฮลิโคแบคเตอร์ ไพโลไร กับโรคแผล เปปติกในผู้ป่วยไทย
แหล่งทุน : เงินทุนวิจัยชีวโมเลกุล คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย และทุนสนับสนุนวิทยานิพนธ์และกลุ่มวิทยานิพนธ์ จุฬาลงกรณ์มหาวิทยาลัย
สถานภาพ : ทำลุล่วงแล้ว 90%

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

ประวัติผู้วิจัย

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4. หน่วยงานที่อยู่ที่สามารถติดต่อได้สะดวก พร้อมหมายเลข โทรศัพท์ โทรสาร และ e-mail
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วิทยาศาสตรบัณฑิต (เทคนิคการแพทย์) จุฬาลงกรณ์มหาวิทยาลัย, 2517
6. สาขาวิชาที่มีความชำนาญพิเศษ (แตกต่างจากวุฒิการศึกษา) ระบุสาขาวิชา
แบคทีเรียทางการแพทย์
7. ประสบการณ์ที่เกี่ยวข้องกับการบริหารงานวิจัยทั้งภายในและภายนอกประเทศ โดย
ระบุสถานภาพในการทำการวิจัยว่าเป็นผู้อำนวยการแผนงานวิจัย หัวหน้าโครงการวิจัย
หรือผู้ร่วมงานวิจัย
 - 7.1. หัวหน้าโครงการวิจัย : ชื่อโครงการวิจัย
 - การเปรียบเทียบวิธี Disk diffusion test , Double disk synergy test และ Three dimensional test ในการหา Extended- Spectrum beta- lactamases ในเชื้อ *Escherichia coli* และ *Klebsiella spp.* ที่แยกได้จากผู้ป่วยในร.พ.จุฬาลงกรณ์
 - การตรวจและจำแนกชนิดเอนไซม์ Extended-Spectrum beta-lactamases สร้างโดยเชื้อ *Escherichia coli* และ *Klebsiella spp.* ที่แยกได้จากผู้ป่วยในโรงพยาบาลจุฬาลงกรณ์
 - 7.2. ผู้ร่วมโครงการวิจัย : ชื่อโครงการวิจัย
 - การสร้างชุดทดสอบสำหรับตรวจสปิซิสของ *Mycobacterium* ด้วย reverse hybridization
 - การเปรียบเทียบวิธีตรวจหาความไวรับต่อยา clarithromycin ของเชื้อ

Mycobacterium avium complex

7.3. งานวิจัยที่ทำเสร็จแล้ว

- 1) Hirunwiwatkul P, Tumwasorn S, Udomsantisuk N, Sirichai N.
Comparative studies of diagnostic tests of tuberculous lymphadenitis:
Culture for TB (BACTEC), Polymerase chain reaction and Histopathology.
Chula Med J 2002 Aug ;46(8) : 619-629
- 2) Tumwasorn S, Nilgate S, Udomsantisuk N. Amplification of P1 Gene by
Polymerase chain reaction for detection of *Mycoplasma pneumoniae*. J Med
Assoc Thai 2002; 85 Suppl1: S389-398

7.4. งานวิจัยที่กำลังทำ

- การเปรียบเทียบวิธี Disk diffusion test , Double disk synergy test และ Three dimensional test ในการหา Extended- Spectrum beta- lactamases ในเชื้อ *Escherichia coli* และ *Klebsiella spp.* ที่แยกได้จากผู้ป่วยในโรงพยาบาลจุฬาลงกรณ์
แหล่งทุน : เงินทุนวิจัยรัชดาภิเษกสมโภช คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
สถานภาพ : ทำลุล่วงแล้ว 90%
- การตรวจและจำแนกชนิดเอนไซม์ Extended-Spectrum beta-lactamases สร้างโดยเชื้อ *Escherichia coli* และ *Klebsiella spp.* ที่แยกได้จากผู้ป่วยในโรงพยาบาลจุฬาลงกรณ์
แหล่งทุน : เงินทุนวิจัยชีวโมเลกุล คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
สถานภาพ : ทำลุล่วงแล้ว 95%
- การสร้างชุดทดสอบสำหรับตรวจสปีชีส์ของ *Mycobacterium* ด้วย reverse hybridization
แหล่งทุน : ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ
สถานภาพ : ทำลุล่วงแล้ว 70%
- การเปรียบเทียบวิธีตรวจหาความไวรับต่อยา clarithromycin ของเชื้อ *Mycobacterium avium* complex
แหล่งทุน : เงินทุนวิจัยรัชดาภิเษกสมโภช คณะแพทยศาสตร์จุฬาลงกรณ์มหาวิทยาลัย และทุนสนับสนุนวิทยานิพนธ์และกลุ่มวิทยานิพนธ์ จุฬาลงกรณ์มหาวิทยาลัย
สถานภาพ : ทำลุล่วงแล้ว 80%

ประวัติผู้วิจัย

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แพทยศาสตรบัณฑิต จุฬาลงกรณ์มหาวิทยาลัย, 2536
วุฒิบัตรแสดงความรู้ความชำนาญทางอายุรศาสตร์ แพทยสภา, 2542
วิทยาศาสตรมหาบัณฑิต (วิทยาศาสตร์การแพทย์) จุฬาลงกรณ์มหาวิทยาลัย, 2544
วุฒิบัตรแสดงความรู้ความชำนาญทางอายุรศาสตร์โรคระบบ การหายใจและภาวะ
วิกฤต ราชวิทยาลัย อายุรแพทย์แห่งประเทศไทย, 2544
หนังสืออนุมัติแสดงความรู้ความชำนาญทางเวชบำบัดวิกฤต ราชวิทยาลัยอายุร
แพทย์แห่งประเทศไทย, 2545
ประกาศนียบัตรแสดงความรู้ความชำนาญทางด้าน acute lung injury จาก Johns Hopkins
Medical Institute, USA, 2547
6. สาขาวิชาที่มีความชำนาญพิเศษ (แตกต่างจากวุฒิการศึกษา) ระบุสาขาวิชา
Pleural diseases
Tuberculous pleuritis
Endothelial cell biology and pathogenesis of acute lung injury
7. ประสบการณ์ที่เกี่ยวข้องกับการบริหารงานวิจัยทั้งภายในและภายนอกประเทศ โดย
ระบุสถานภาพ ในการทำการวิจัยว่าเป็นผู้อำนวยการแผนงานวิจัย หัวหน้า
โครงการวิจัย หรือผู้ร่วมงานวิจัย
7.1. หัวหน้าโครงการวิจัย : ชื่อโครงการวิจัย

- Differential Regulation of Human Lung Epithelial and Endothelial Barrier Function by Thrombin.
- Unplanned Extubation in Medical ICU
- Polymerase Chain Reaction of Pleural Biopsy Specimens for Diagnosing Tuberculous Pleuritis.

7.2. ผู้ร่วมโครงการวิจัย : ชื่อโครงการวิจัย

- Epoxycyclopentenone-containing oxidized phospholipids restore endothelial barrier function via Cdc42 and Rac.
- Differential effects of shear stress and cyclic stretch on focal adhesion remodeling, site-specific FAK phosphorylation and small GTPases in human lung endothelial cells.
- Magnitude-Dependent Regulation of Lung Endothelial Barrier by Cyclic Stretch: Role of Rho and Rac

7.3. งานวิจัยที่ทำเสร็จแล้ว

- 1) Differential Regulation of Human Lung Epithelial and Endothelial Barrier Function by Thrombin. **Kawkitinarong K**, Linz-McGillem L, Birukov KG, Garcia JG. Am J Resp Cell Mol Biol. 2004 Nov; 31(5):517-27.
- 2) Epoxycyclopentenone-containing oxidized phospholipids restore endothelial barrier function via Cdc42 and Rac. Birukov KG, Bochkov VN, Birukova AA, **Kawkitinarong K**, Rios A, Leitner A, Verin AD, Bokoch GM, Leitinger N, Garcia JG. Circ Res 2004 Oct 29; 95(9): 892-901.
- 3) Differential effects of shear stress and cyclic stretch on focal adhesion remodeling, site-specific FAK phosphorylation and small GTPases in human lung endothelial cells. Shikata Y, Rios A, **Kawkitinarong K**, DePaola N, Garcia JG, Birukov KG. Exp Cell Res 2004 (in press).
- 4) Unplanned Extubation in Medical ICU นี้อต เตชะวัฒนวรรณ, ฉันทชาย สิทธิพันธ์, สมเกียรติ วงษ์ทิม, วิศิษฐ์ อุคตพาณิชย์, กมล แก้วกิตติรงค์ วารสารวันโรค โรคทรวงอก และเวชบำบัดวิกฤต 2547 (อยู่ระหว่างตีพิมพ์)
- 5) Polymerase Chain Reaction of Pleural Biopsy Specimens for Diagnosing Tuberculous Pleuritis. (in preparation).

7.4. งานวิจัยที่กำลังทำ

การศึกษาประสิทธิภาพของ PCR ในการวินิจฉัยวัณโรคเชื้อหุ้มสมอง