RRPG91120311 (32 .P)

計畫編號：DOH92-DC-1052

行政院衛生署九十二年度科技研究發展計畫

利用聚合酵素連鎖反應及變性高效能層析法來快速偵測抗藥性結核菌

成 果 報 告

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執行期間：92 年 1 月 1 日至 92 年 12 月 31 日

＊＊本研究報告僅供參考，不代表本署意見＊＊

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中文摘要

臺灣地區由結核桿菌所造成的感染有逐年增加的趨勢，且居國內傳染病之首位。最近，由多重抗藥性之結核桿菌(Multidrug resistant *Mycobacterium tuberculosis*)所引起的群突發(outbreak)，其致死率高達 72-89%，且病人從診斷到死亡之間卻只有短短地 4-16 週，再加上目前結核桿菌及其抗藥性試驗檢驗曠日費時，因此在當今由結核桿菌造成的高致死率以及如此快速的發病過程的情況下，發展一套快速偵測多重抗藥性結核桿菌之方法已成為當下刻不容緩的工作。Isoniazid (INH), rifampin (RIF), streptomycin (SM)是臨床上對抗結核桿菌的常用第一線藥物。目前研究顯示，多重抗藥性之結核桿菌在此第一線藥物所作用的基因上有突變的現象，這些突變基因可藉由聚合酵素連鎖反應(polymerase chain reaction, PCR)及 DNA 定序結果，來快速偵測多重抗藥性結核桿菌。DHPLC (Denaturing high performance liquid chromatography)變異性高效能層析方法乃利用離子配對逆向層析及溫控式異型雙股分析系統 (Temperature Modulated Heteroduplex Analysis, TMHA)原理來進行突變和單核苷酸多形性 (Single Nucleotide Polymorphisms, SNP)之檢測。本計畫之目的乃在建立並利用此 DHLPC 技術來偵測結核桿菌之藥物所作用的基因上有否突變，進而能快速診斷出多重抗藥性之結核桿菌。本計畫一共收集 80 株抗藥性結核桿菌，經由 PCR 增幅抗藥基因片段以及 DHPLC 分析之後，與核酸定序結果做比對，確實可以快速偵測到基因突變的菌株，因此本計畫已發展出一套快速的分子診斷方法，可增快對於結核桿菌抗藥菌株之偵測，對於病人的醫療與照顧具有相當大的貢獻。

關鍵詞：多重抗藥性之結核桿菌(Multidrug resistant *Mycobacterium tuberculosis*)、抗藥基因(resistant genes)、變異性高效能層析法(Denaturing high performance liquid chromatography, DHPLC)

ABSTRACT

In Taiwan, the infections of *Mycobacterium tuberculosis* increased gradually year by year. Meanwhile, the *Mycobacterium tuberculosis* was the most common agent for clinical infections. Recently, the outbreak of multidrug resistant *Mycobacterium tuberculosis* (MDRTB) can cause the mortality of 72-89% and it was just 4-16 weeks from diagnosis to death. Furthermore, it is time consuming for the antibiotic susceptibility of *Mycobacterium tuberculosis*. Thus, it was the first case to develop a diagnostic method for detection of MDRTB under the threat of high mortality and fast course of infection. Isoniazid (INH), rifampin (RIF), and streptomycin (SM) are the first line anti-TB drugs. In present, there are mutations at the resistant genes in MDRTB. We can detect the MDRTB strains quickly by using the PCR and DNA sequencing methods followed by denaturing high performance liquid chromatography (DHPLC) and temperature modulated heteroduplex analysis (TMHA). The purpose of this project is to develop a technique to diagnose the MDRTB strains by using DHPLC analysis to detect the mutation pattern of resistant genes. We totally collected 80 MDRTB strains. After PCR amplification of resistant gene fragment, DNA sequencing, and DHPLC analysis, the mutant strains can be detected fast and accurately. To sum up, this project had developed a fast molecular diagnostic method to detect the MDRTB strains and it had a great benefit to the patients for the good medical care.

Keywords： Multidrug resistant *Mycobacterium tuberculosis*, MDRTB; resistant genes; Denaturing high performance liquid chromatography, DHPLC)

1. 前言

結核病是目前全球各種傳染病中引起最多死亡的疾病。根據估算：全球大約已有 1/3 人口感染結核菌，每一秒就有一人感染結核，每年約有 1%人口會感染結核菌；目前這些已受感染者，此生約有 5～10%的機會發病。由於結核防治計畫不完善、愛滋病盛行及全球人口快速流動，更加速了結核病的傳播。又根據先前世界衛生組織的統計：1990 年全世界約有 750 萬新發病之結核人（每十萬人口 143 人），約有 50 萬人死於結核病；到了公元 2000 年，更高達 1020 萬新發現之結核病人（每十萬人口 163 人），且將會有 350 萬人死於結核病。臺灣地區由結核桿菌所造成的感染也有逐年增加的趨勢，且居國內傳染病之首位。根據台灣衛生署 2000 年的統計，平均每天新增 38 位結核病患，平均每天約 4.20 人死於結核病。由於不完整療程或不當使用藥物而產生的次發性抗藥結核桿菌也直線上升。在台灣地區，多種抗藥性結核桿菌(Multiple drug resistant *Mycobacterium tuberculosis*, MDRTB)產生比例約有 23%(Yu et al., 1994)，而美洲地區的比例約為 26.6% (Bloch et al., 1994)。

多重抗藥性結核桿菌(MDRTB)與原來的野生型結核桿菌(wild type *M. tuberculosis*)有很大差異，不但造成高死亡率(約 80%)，且病程惡化迅速,從診斷至死亡僅需 4–16 週(Dooey et al., 1992; Fischl et al.,1992; Freidan et al.,1993)。此外多重抗藥性結核桿菌具有高傳染力，尤其是對與這類病人同居一室的其他患者及負責照顧的醫護人員更是一大威脅。國外學者研究報告中發現,此型結核桿菌對照顧的醫護人員傳染率達 34%(Dooey et al.,1992)

MDR-TB 之抗藥分子機轉

在多種抗藥型結核桿菌的分子生物學研究中發現,其抗藥性可能源自染

色體，對不同類藥物的抗藥性機轉，主要是各自獨立的基因發生突變(Then et al.,1983; Iseman & Madsen, 1989; David, 1980)。

Isoniazid(INH)

INH 是在抗結核藥物中最普通使用的藥物之一，但其對結核桿菌之作用機轉到目前並不完全瞭解，初步瞭解對 INH 產生的抗藥性是在 catalase-peroxidase genes(*katG*)發生突變，或是在 inhA 基因發生突變；而此兩種基因是 mycolicacid 之合成有關(Zhang et al., 1992; Banerjee et al.,1994)。研究報告中發現將帶有完整 *kat*G 基因轉形至抗 INH 結核桿菌株，會使此抗 INH 菌株變成對 INH 有感受性(Zhang et al.,1992&1993)。其他研究顯示，將錯意義突變(missense mutation)之 *inh*A 基因轉形至有 INH 感受性之 *M. bovis* 和 *M. smegmatis*，會使其變成有抗藥性(Banerjee et al.,1994).。CookerillIII 及 Morris 等學者研究發現有 44%以上之抗 INH 結核桿菌株在 *kat*G 基因第 463 codon 位置，其 Arginine 會變成 Leucine 之現象。有 10-25%在第 315 codon 位置，其 Serine 會變成 Threonine。Banerjee 等人也報告抗 INH 之結核桿菌在 *inh*A 基因第 94 codon，其 Serine 變成 Alanine。

Rifampin (RMP)

RMP 是一種複合 macrocyclic 抗生素，藉由與 DNA-dependent RNA polymerase 結合而抑制細菌 RNA 之合成。在大部分抗 RMP 結核桿菌之分子研究顯示，其抗 RMP 乃由於譯成 RNA polymerase 之 β-subunit 的基因(*rpo*B)發生錯意義突變。研究發現有 90%以上之抗 RMP 結核桿菌在此 *rpo*B 基因之 69bp 位置有發生突變。而此 23 個胺基酸最常發生突變位置依序為第 531 (serine), 526 (histidine)和 516 (aspartic acid) (Morris et al, 1995; Telenti et al.,1993a; Miller et al., 1994; Kapur et al., 1994; Williams et al.,

1994)。由於如此少的點發生突變與 RMP 是抗藥性有關，就可發展出一利用聚合酵素鏈反應技術簡單又快速的方法來篩選造成突變的基因片段是否存在。這樣的技術結合前述偵側 INH 是抗藥性的方法，將可快速偵側出具 MDRTB 菌株來。

Streptomycin (SM)

SM 是一種 aminoglycoside 抗生素，可干擾細菌之蛋白質合成。由於它可與 16S 核糖體 RNA (rRNA)結合，而引起基因密碼之誤讀(misreading)和抑制轉譯啟始(translational initiation) (Finken et al., 1993; Nair et al., 1993; Meier et al., 1994; Honore & Cole, 1994)。研究發現對 SM 抗藥性的發生，是結核桿菌內控制核糖體蛋白 S12(ribosomal protein S12)的基因(*rpsL*)或是在控制 16S 核糖體核糖核酸(16S ribosomal RNA, 16S rRNA)基因(*rrs*)發生突變，其中以 *rpsL* 基因發生錯意義突變為最多(Morris et al., 1995)，有 50%在 codon 43 會有 transition 由 A 變 G，7%在 codon 88 有同樣突變。此外，有 10%抗 SM 結核桿菌在 16S 核糖體 RNA 之 513 位置也有突變。且大於 75%抗 SM 結核桿菌株會在 *rpsL* 或 *rrs* 基因上有突變(Morris et al., 1995; Finken et al., 1993)。到目前為止，無任何 SM 感受性結核桿菌顯示在此二基因有突變現象。

其他抗結核藥物

Pyrazinamide(PZA)是一種 nicotinamide 合成物，是治療結核病之短程藥物，其可經由細胞內的 pyrazinamidase 或 nicotinamidase 作用成 pyrazinoicacid。目前研究發現，控制 pyrazinamidase 或 nicotinamidase 之基因(*pncA*)若失去或發生 missense mutation，則會導致結核桿菌對 PZA 產生抗藥性(Scorpio and Zhang, 1996)。Ethambutol(EMB)是一種非常特異和有效的

藥，它通常與 INH 一起來治療結核病，其可抑制 D-arabinase 變成 arabinozalactan，目前 EMB 之分子機轉尚未知。有關 quinolones 藥物，Takiff 等人發現結核桿菌中 gyrase A 基因(*gyr*A)突變會造成對 fluroguinolones 產生高度抗藥性，至於低抗藥性機轉仍不清楚。

目前結核桿菌抗藥基因臨床應用

過去一般認為除非是抗藥菌盛行地區，對初次分離的結核菌作藥物感受性試驗是不切實際的(American Thoracic Society, 1974; Base et al., 1990)。近年來由於 MDRTB 之出現，情況有所改變。所有初次分離菌都要試驗其藥物感受性(Tenover et al., 1993)。

目前傳統的檢驗室診斷抗藥性結核菌方法耗時甚久，由收集檢體到結核桿菌培養鑑定至少要 4–6 週；若須抗藥性確定而進行藥物敏感度試驗，再要 2–4 週；總共需要 6–10 週 (Kent & Kubica, 1985)。此種診斷方法對於臨床治療需要迅速的原則幫助不大，及太慢提供訊息以供醫護人員自我保護及隔離。近年發展自動化的 BACTEC 偵測法(Middlebrook et al.,1977)，對主要的抗結核藥物，在五日內即可完成藥物感受性試驗(Huebner et al., 1993; Goble, 1986; Tenover et al.,1993)。然而由於 BACTEC 系統是採用 C^{14} 放射元素來偵測，在臨床上應用有所困難。

近年來由於分子生物學技術的進步，使得 MDRTB 診斷技術有所改善。目前以 PCR 技術來偵測 MDRTB 之菌株，也有數十篇以上報告過。其基本原理乃取決於對藥物呈敏感性及抗藥性的菌株中，遺傳物質間的潛在差異性(Dooley et al., 1992; Morris et al., 1995)。利用 PCR 技術，將此段差異加以增幅(amplification)，然後再以特殊電泳系統分析(如 single strand conformational polymorphism, SSCP 或 denaturing gradient gel

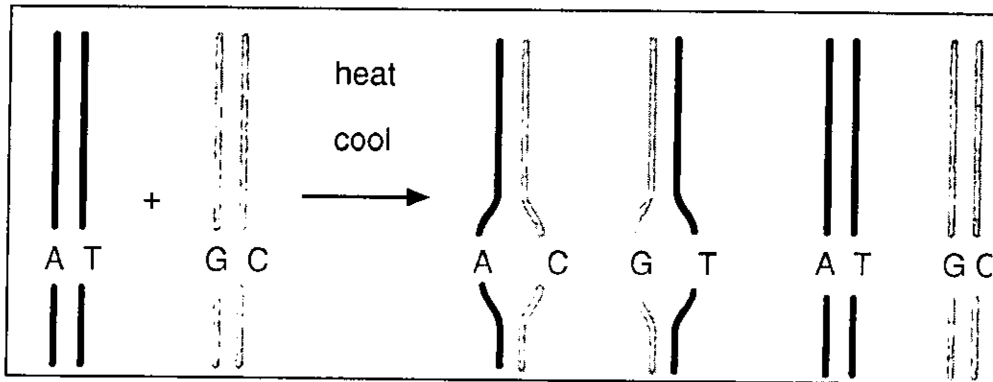
electrophoresis, DGGE)，或是以自動DNA序列分析其間的差異，如此以區別敏感性及抗藥性之結核菌株(Telenti et al.,1993a & 1993b)。本計畫即以特殊核酸分離系統(DHPLC)來快速偵測基因的突變情形。

DHPLC之原理與其應用

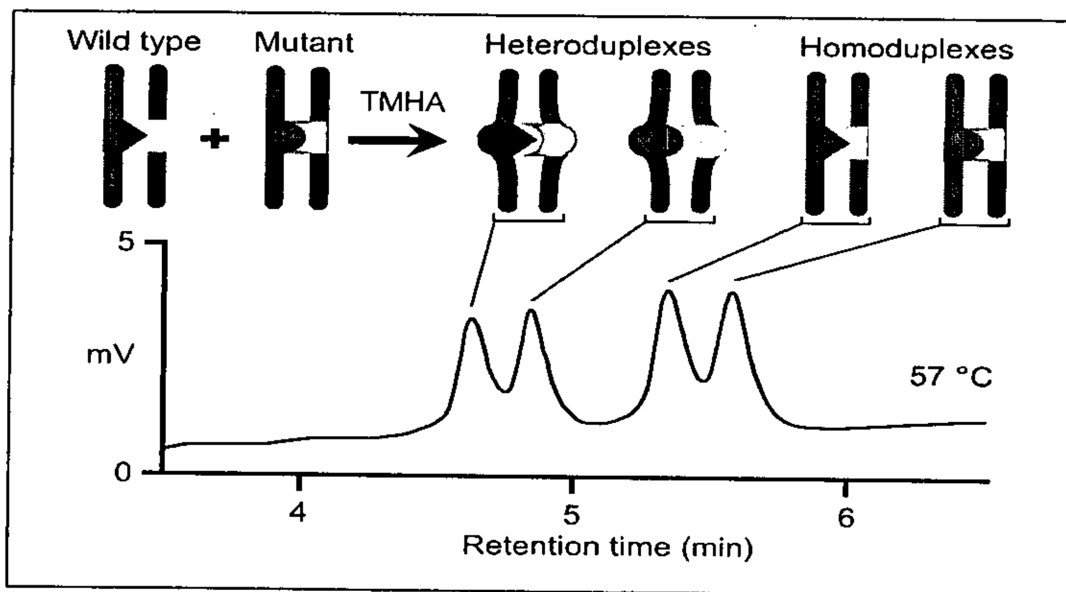
DHPLC (Denaturing high performance liquid chromatography)變異性高效能層析方法乃利用離子配對逆向層析及溫控式異型雙股分析系統(Temperature Modulated Heteroduplex Analysis, TMHA)原理來進行突變和單核苷酸多形性 (Single Nucleotide Polymorphisms, SNP)之檢測。這技術為美國 Standford 大學 Oefner 和 Underhill (1998)博士所開發，目前由美國 Transgenomic 公司所研發出稱之為 Wave system。所謂離子配對逆向層析原理是利用離子配對試劑 TEAA (triethyl-ammonium acetate)與 DNA 的磷酸基形成離子配對，在流動相中帶正電的 TEAA 會將表面帶負電 DNA 包覆，並以非極性的烷基端吸附在非極性的固定相上，此非極性的固定相指分離管柱的基質藉由化學鍵結合有 18 個碳的烷基，雙股 DNA 在分離管柱的滯留主要決定於 TEAA 中帶正電離子和 DNA 中帶負電離子的結合力，若 DNA 鏈越長，就帶有越多磷酸基，因此有較多的 TEAA 結合使其在管柱滯留的時間越長，藉由增加流動相中 Acetonitrile 濃度使具有親水性的 Acetonitrile 將 DNA 置換就是造成 DNA 和 TEAA 的脫附，進而使 DNA 分子隨流動相流出而達到分離效果。

一般雙股 DNA 在適當局部變性溫度下藉由分離管柱能靈敏地檢測到一帶有單一鹼基錯配的核酸片段，例如選取只有單一鹼基變異而其他序列均相同的兩個雙股 DNA 進行 PCR 後各取等量比例混合，經由溫度上升造成兩個雙股 DNA 打開成四個單股，再經由溫度下降過程中四個單股 DNA 會重新配對產生兩大類共四種的雙股 DNA 分子，包括兩種序列完全配對的同

質雙股螺旋(homoduplexes)和兩種是具有單一鹼基錯配的異質雙股螺旋(heterodulexes)，如下圖：



此四種 DNA 分子在增溫管柱中進行分析時，在未達到該 DNA 分子的變性溫度前(如 50℃)，完全配對的同質雙股螺旋和具有單一鹼基錯配的異質雙股螺旋是無法被區分的，在層析圖上始終呈現一個吸收峰，若在理想的變性溫度中(如 56℃)，四種 DNA 分子在增溫管柱中進行分析時，層析圖上顯示為四支明顯吸收峰，表示有四種物種存在於 DNA 樣品中，具有單一鹼基錯配的異質雙股螺旋總是在序列完全配對的同質雙股螺旋之前被流析出，四種 DNA 物種被流析出的順序依據其熱穩定度的排列順序(如下圖)，此即所謂溫控式異型雙股分析系統。



DHPLC 能在短時間內偵測核酸片段中單一核酸的變異，並且可以快速分析小片段鹼基插入(Insertion)和缺失(Deletion)，平均每一樣品所花時間約 10 分鐘，分析片段可達 1000bp，比傳統以凝膠電泳技術方式縮短分析時間且增加檢測靈敏度，並且經過 PCR 所產生的核酸片段不須要經由純化步驟就能進樣分析。

近來文獻報導 DHPLC 系統具有相當高的靈敏度，Holiski-Feder 於 2001 發表用 DHPLC 分析遺傳性非瘜肉大腸癌中 MLH1 和 MSH2 基因，其靈敏度高達 97%。O'Donovan 於 1998 年發表 Factor IX 凝血因子 H 的盲樣分析指出 DHPLC 有 100%靈敏度。Gross (1999)等人分析 BRCA1 突變情形，指出 DHPLC 有 100%靈敏度和專一性。Wagner(1999)等人指出 DHPLC 的成本只有定序的十分之一主要節省下是試劑和勞力的成本。Liu 等人於 1998 年從 63 個神經膠質瘤發現 18 個新的體細胞 PTEN 基因突變，DHPLC 的檢測靈敏度為 95%。

由於結核桿菌在全世界的再現，導致醫療資源超支，尤其 MDRTB 引發驚人的罹患率、死亡率及醫療照顧人員被感染等問題，再加上目前結核桿菌的抗藥性試驗曠日費時，發展一套快速偵測多重抗藥性結核桿菌之方法已成為當下刻不容緩的工作。本計劃希望能利用 DHPLC 快速鑑定結核桿菌中抗藥性基因檢測，以提供給臨床醫生作為治療用藥之參考。

2.材料與方法

本計劃在執行期間，從三軍總醫院及台北市立慢性病防治院的臨床檢體中收集 80 株多重抗藥性結核桿菌菌株。利用聚合酵素連鎖反應（PCR）方法來增幅在些菌株中的 *katG*, *inhA*, *rpoB*, *rpsL* 及 *rrs* 基因，然後進行核酸定序與 DHPLC 來分析這些 PCR 產物。

根據分析定序結果，可以確定結核桿菌抗藥性菌株的突變情形，再與 DHPLC 結果進行比較，可以瞭解 DHPLC 技術在偵測各種抗結核藥物作用基因突變的能力。

細菌菌株：A total of 80 MDRTB strains from clinical isolates from Tri-Service General Hospital and Taipei Municipal Chronic Disease Hospital were collected. Strains of *M. tuberculosis* (ATCC25618-H37RV, ATCC35838-H37RV-RIF, ATCC35822H37RV-INH) were used as the wild- and mutant-type reference strain. Homogeneity of drug resistance was ensured by culturing the resistant strains on Middlebrook 7H10 agar containing the following drugs at the indicated concentrations: rifampin (1 μg/ml); isoniazid (1 μg/ml) and streptomycin (10 μg/ml).

細菌染色體 DNA 純化：Bacterial genomic DNA was prepared by extraction with phenol and chloroform as described previously (Santos et al., 1992). Briefly, tubercle bacilli were washed from Lowenstein-Jensen and Middle brook agar slants with Long's synthetic medium and centrifuged at 12,000 x g for 5 min. After supernatant removed, cells were resuspended with 0.5 ml TE buffer (10 mM Tris [pH8.0], 1 mM EDTA). Then, 50 μL of 10 mg/mL lysozyme was added and incubated at 37°C for 1 hr. The reaction solution was added with 60 μL of 10% SDS, and 6 μL of 20 mg/ml of proteinase K, and

incubated at 65°C for 10 min. The digests were mixed with equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) by vortexing the tube for 30 sec and centrifuged at 12,000 x g for 5 min. The aqueous phase was transferred into a new microcentrifuge tube, and DNA was precipitated with 1/10 volume of 3M sodium acetate and 1 volume of 100% isopropanol. DNA was pelleted at 12,000 x g for 10 min. The pellet was washed with 0.5 mL of 70% ethanol and air dried. The dried pellet was resuspended with 100 μL of TE buffer. The purified DNA was ready for PCR amplification.

PCR 增幅反應： The PCR mix contained template DNA, PCR buffer (10 mM Tris-HCl, pH 8.8 at 25°C; 1.5 mM $MgCl_2$; 50 mM KCl; 0.1% Triton X-100), 0.2 μM of each PCR primer, 0.2 mM of each dNTP and 1.0 U of DyNAzyme™ II DNA Polymerase (Finnzymes Oy, Espoo, Finland) in a total volume of 50 μL. The primer sets and PCR conditions were used to amplify genes associated with drug resistance are listed in Table 1.

PCR 產物純化： The PCR products were purified using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences Corp, NJ, USA) according to the manufacturer's instructions. Briefly, add 500 μL of capture buffer and transfer the PCR product (up to 100 μL) to the GFX column. Mix thoroughly by pipetting the sample up and down 4–6 times. Centrifuge at full speed for 30 sec and discard the flow-through by emptying the collection tube. Add 500 μL of wash buffer to wash the column and apply 50 μL of elution buffer (10 mM Tris-HCl pH 8.0, TE pH 8.0) to elute DNA.

核酸定序： Sequencing reactions with BigDye terminator cycle sequencing kit (Applied Biosystems Inc.) were performed with 30 ng of purified PCR-amplified DNA as template and 3.2 pmole of appropriate sequencing

primers. The whole procedures were performed as according to the manufacturer's instructions. After sequencing reaction, the excess dye terminators were removed by ethanol precipitation, then the products were dried in a vacuum centrifuge, resuspended in 6 μL of loading buffer (5:1deionized formamide-50 mM EDTA [pH8.0]), heated denatured for 2 min at 90°C, and immediately loaded on an 5% Long Ranger gel in an Applied Biosystems model 377 automated sequencer.

DHPLC分析： DHPLC analysis was carried out on an automated HPLC device equipped with a DNA separation column (WAVE: Transgenomic, San Jose, CA, USA). Four to 7 μL of each PCR product (containing 50-100 ng of DNA) was denaturated for 3 min at 95°C and then gradually reannealed by decreasing the sample temperature from 95 to 65°C over a period of 30 min. PCR products were then separated at a flow rate 0.9 mL/min by means of a linear acetonitrile gradient. Gradient parameters were determined based on size and G+C content of the amplicon. Generally, analysis was taken approximately 7 min including column regeneration and re-equilibration to starting conditions. The column mobile phase consisted of a mixture of 0.1 M triethylamine acetate (pH 7.0) with (buffer B) or without (buffer A) 25% acetonitrile. Temperature for successful resolution of heteroduplex molecules was determined by running fragment-specific melting curves and by using the DHPLC melting algorithm WAVEE Maker of the WAVEE instrument. Melting curves were determined as follows: the elution time of a specific fragment under standard conditions was determined. This specific gradient was then tested for the same PCR product for temperatures ranging from 48 to 70°C, and retention time versus temperature was graphed to yield a fragment-specific melting curve.

3.結果

3.1 菌株收集

本計劃執行期間，從三軍總醫院及台北市立慢性病防治院的臨床檢體中，收集 80 株多重抗藥性結核桿菌菌株。針對四種(INH, RIF, STR, EMB)抗結核桿菌藥物，以傳統比例法進行抗生素敏感性試驗，結果顯示，INH 的抗藥率為 51%，RIF 的抗藥率最高為 78%，其次是 STR 的抗藥率為 65%，EMB 具有最低的抗藥率為 8% (Table 1)。

3.2 抗藥基因序列分析

總共 80 株菌株，進行四個抗藥基因的 PCR 產物直接定序工作，先以 forward primer 進行定序，經過序列比對之後，若發現有突變的位點，則該位點會以 reverse primer 定序確定之。

所有定序比對結果找到的突變位點及菌株的統計結果，綜合於 Table 2。*katG* 基因在 codon 315 有兩種突變(S315T, S315N)，所佔的比例是 73% (30/41)，有 27% (11/41)未發現突變；*rpoB* 基因的突變點較多，其中 S531L 與 H526D 的頻率較高，分別為 18% (11/62)與 13% (8/62)，有 32% (20/62)未發現突變；*rpsL* 基因在 K88R 與 K43R 兩處有突變，其頻率依序為 29% (15/52)與 19% (10/52)，有 52% (27/52) 未發現突變；*embB* 基因在 codon 306 同一處有兩種突變(M306V, M306I)，所佔的比例是 83% (5/6)，有 17% (1/6)未發現突變。

3.3 DHPLC分析

利用WAVE program預測各個抗藥基因PCR片段之melting point，然後再實際上機測試DHPLC之作用溫度，測得的溫度列於

Table 4.

於Figure 1中顯示*katG*基因進行DHPLC之結果層析圖，G2922C(S315T)與G2922A (S315N)確實可以跟沒有突變的菌株區分出來，可是由於位點相同，層析圖譜的波動樣式並不能區分出此兩者的突變。

於Figure 2中顯示*rpoB*基因進行DHPLC之結果層析圖，由於此基因突變位點較多，突變的圖譜樣式亦較多樣，其中TTC2379–2381 insertion (F514)由於序列上差異較大，因此層析圖譜樣式亦特別明顯；A2385T (D516V)與A2377T (Q513L)具有可觀察到的圖譜樣式，但是卻不夠明顯；另外，C2431T (S531L)與C2415G (H526D)具有獨特的圖譜樣式，可直接從樣式即可判斷出突變形式。

於Figure 3中顯示*rpsL*基因進行DHPLC之結果層析圖，A128G (K43R)與A263G (K88R)具有獨特的圖譜樣式，可直接從樣式即可判斷出突變形式。

於Figure 4中顯示*embB*基因進行DHPLC之結果層析圖，A7868G (M306V)與G7870A (M306I)具有明顯的圖譜樣式，但由於位點過於接近，因此圖譜樣式非常相似，不易從樣式中判斷突變形式。

4.討論

傳統上檢驗室診斷抗藥性結核菌方法耗時甚久，約需要 6–10 週的時間 (Kent & Kubica, 1985)。因此發展一套快速的檢驗技術，對於結核桿菌的預防與治療將有很大的幫助，一般而言，細菌的抗生素敏感性試驗，可分為表現型與基因型檢測方法，近年來由於分子生物學技術的進步，使得有許多基因型檢測法被發展出來，其基本原理乃利用 PCR 的快速增幅 DNA，對於特定基因進行突變的檢測，這樣的檢測會有先天的缺陷，原因在於某一個藥物的抗藥性經常不是決定於單一基因，有可能許多種基因牽涉其中，因此以基因型檢測法可能會有僞陰性的結果發生，這在本計劃的結果中(Table 3)可發現，並不是所有抗藥性菌株都找得到基因的突變點，未發現的比例從 17%至 52%，這樣的結果確實需要加以重視，改進的方法可以增加多種基因的突變偵測，盡量涵蓋所有已發表之抗藥基因。因此如能以 Multiplex DHPLC 系統來同時分析多個產物，將可解決增加抗藥標的基因的問題，又可縮短單一菌株的檢測時間。

由於傳統抗藥性結核菌檢測的方法是以比例法來進行，超過百分之一以上的菌株具有抗藥性，即判定為抗藥性菌株，但是以基因型檢測法進行時，敏感性要達到百分之一是相當困難的，因此有可能會因為敏感性不足而造成僞陰性的結果，例如 DNA 定序就不易觀察到低於 50%的突變族群。本計劃所使用的 DHPLC 系統，其敏感性可以到達 0.5%至 5%左右，這樣的優勢可以彌補 DNA 定序之缺陷。

DHPLC 在實驗技術上必須特別注意的就是 PCR 產物的純度，因此使用具有 hot start 與 proof reading 的 Taq DNA polymerase，可以避免核酸產物的人為變異以及非特異性產物的影響。理論上，不同的突變形式，DHPLC 的層析圖譜樣式就應當有所不同，可是由於分析溫度的設定、突變族群所

佔的比例、核酸產物的純度等均會影響 DHPLC 的結果，因此想要以層析圖譜樣式來推測突變的形式確實有所困難，只可知道有突變產生，至於是否是已知的抗藥性突變，亦或是新的突變，而此新的突變是否會造成抗藥性，或只是 silence mutation，這都要以定序的結果來加以佐證或推測，更甚者，或許需要進行傳統的比例法抗藥性試驗，才可獲得最後的證實。所以除了基因型的檢測方法之外，若能配合表現型的方法，將可彌補以上的缺憾，近年來科學家們亦開發出許多表現型抗藥性試驗，例如 phage amplified biologically (PhaB) assay、E-test、Nitrate reductase assay、Reverse Transcription-PCR、Microplate-based Alamar Blue Assay (MABA) method 、Microscopic Observation Drug Susceptibility Assay 等方法，均可在 7 天左右獲得初步的抗藥性結果，因此若能將基因型與表現型方法互相配合，將可獲得快速正確的抗藥性結果。

5.結論與建議

在本計畫當中，利用定序技術所找到的突變位點，確實均可使用DHPLC系統加以偵測出來，因此利用本計畫所建立的方法，以PCR的快速增幅DNA能力，再配合DHPLC快速敏感地鑑定結核桿菌是否帶有抗藥突變基因，確實可以提早給予臨床醫生治療用藥之參考。

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7.圖表

TABLE 1. PCR primers for amplification of anti-drug resistance genes.

Gene	Protein (drug affected)	Primers (5' >3')	PCR product
katG	Catalase peroxidase (INH)	katG-AF: GGTCGACATTCGCGAGAC katG-AR: AGAGGTCAGTGGCCAGCAT	413
		katG-BF: GAGTGGGAGCTGACGAAGAG katG-BR: CTTGTCGCTACCACGGAAC	447
rpoB	β subunit RNA polymerase (RIF)	rpoB-F: CGACGACATCGACCACTTC rpoB-R: GGTTTCGATCGGGCACAT	364
rpsL	S12 ribosomal protein (STR)	rpsL-F: GCAGCGTCGTGGTGTATG rpsL-R: TCTTGACACCCTGCGTATCC	242
embB	Arabinosyl transferase (EMB)	embB-F: CGCACCTTCACCCTGACC embB-R: AGCAGCAGCCAGCACACTA	275

INH: isoniazid; RIF: rifampin; STR: streptomycin; EMB: ethambutol

TABLE 2. Bacterial Susceptibility to anti-TB drugs.

Susceptibility \ Drugs	Strain No.(%)			
	INH	RIF	STR	EMB
Resistant	41 (51)	62 (78)	52 (65)	6 (8)
Sensitive	39 (49)	18 (22)	28 (35)	74 (92)

TABLE 3. DNA sequence analysis for anti-drug resistance genes.

Gene	Protein (drug affected)	Nucleotide (amino acid) of polymorphism	Strains	Total (Resistant)
katG	Catalase peroxidase (INH)	None	11	41
		G2922C(S315T)	13	
		G2922A (S315N)	17	
rpoB	Beta subunit RNA polymerase (RIF)	None	20	62
		A2377T (Q513L)	3	
		TTC2379–2381 insertion (F514)	1	
		A2385T (D516V)	4	
		AAC2390–2392 deletion(N518)	1	
		CA2415–16TG (H526C)	3	
		C2415T(H526Y)	6	
		C2415G (H526D)	8	
		C2431 T(S531L)	11	
		T2437C(L533P)	5	
rpsL	S12 ribosomal protein (STR)	None	27	52
		A128G (K43R)	10	
		A263G (K88R)	15	
embB	Arabinosyl transferase (EMB)	None	1	6
		A7868G (M306V)	3	
		G7870A(M306I)	2	

INH: isoniazid; RIF: rifampin; STR: streptomycin; EMB: ethambutol

TABLE 4. The sequence of PCR fragment and temperature for DHPLC analysis

Gene	Protein (drug affected)	PCR fragment sequence	DHPLC analysis Temp.
katG	Catalase peroxidase (INH)	GGTCGACATTCGCGAGACgtttcggcgcatggccatgaacgacg tcgaaacagcggcgctgatcgtcggcggtcacactttcggtaagacccatggcg ccggcccggccgatctggtcggccccgaacccgaggctgctccgctggagca gatgggcttgggctggaagagctcgtatggcaccggaaccggtaaggacgcga tcaccAGCggcatcgaggtcgtatggacgaacaccccgacgaaatgggaca acagtttcctcgagatcctgtacggctacgagtgggagctgacgaagagccctgc tggcgcttggcaatacaccgccaaggacggcgccggtgccggcaccatcccgg acccgttcggcgggccagggcgctccccgacgATGCTGGCCACTG ACCTCT	60°C
		GAGTGGGAGCTGACGAAGAGccctgctggcgcttggcaatac accgccaaggacggcgccggtgccggcaccatcccggacccgttcggcgggc cagggcgctccccgacgatgctggccactgacctctcgctgcgggtggatccga tctatgagcggatcacgcgtcgctggctggaacaccccgaggaattggccgacg agttcgccaaggcctggtacaagctgatccaccgagacatgggtcccgttgcga gataccttgggccgctggtccccaagcagaccctgctgtggcaggatccggtcc ctgcggtcagccacgacctcgtcggcgaagccgagattgccagccttaagagcc agatccgggcatcgggattgactgtctcacagctagtttcgaccgcatgggcggc ggcgtcgtcGTTCCGTGGTAGCGACAAG	62°C
rpoB	β subunit RNA polymerase (RIF)	CGACGACATCGACCACTTCggcaaccgccgcctgcgtacggtc ggcgagctgatccaaaaccagatccgggtcggcatgtcgcggatggagcgggt ggtccgggagcggatgaccacccaggacgtggaggcgatcacaccgcagacg ttgatcaacatccggccggtggtcgccgcgatcaaggagttcttcggcaccagcc agctgagccaattcatggaccagaacaacccgctgtcggggttgacccacaagc gccgactgtcggcgctggggcccggcggtctgtcacgtgagcgtgccgggctg gaggtccgcgacgtgcacccgtcgcactacggccggATGTGCCCGAT CGAAACC	59°C
rpsL	S12 ribosomal protein (STR)	GCAGCGTCGTGGTGTATGcacccgcgtgtacaccaccactccga agaagccgaactcggcgcttcggaaggttgcccgcgtgaagttgacgagtcag gtcgaggtcacggcgtacattcccggcgagggccacaacctgcaggagcactc gatggtgctggtgcgcggcggccgggtgaaggacctgcctggtgtgcgctaca agatcatccgcggttcgctGGATACGCAGGGTGTCAAGA	56°C
embB	Arabinosyl transferase (EMB)	CGCACCTTCACCCTGACCgacgccgtggtgatattcggcttcctg ctctggcatgtcatcggcgcgaattcgtcggacgacggctacatcctgggcatgg cccgagtcgccgaccacgccggctacatgtccaactatttccgctggttcggcag cccggaggatcccttcggctggtattacaacctgctggcgctgatgacccatgtca gcgacgccagtctgtggatgcgcctgccagacctggccgccgggcTAGTG TGCTGGCTGCTGCT	58°C

Figure 1: DHPLC detection of *katG* gene mutations.

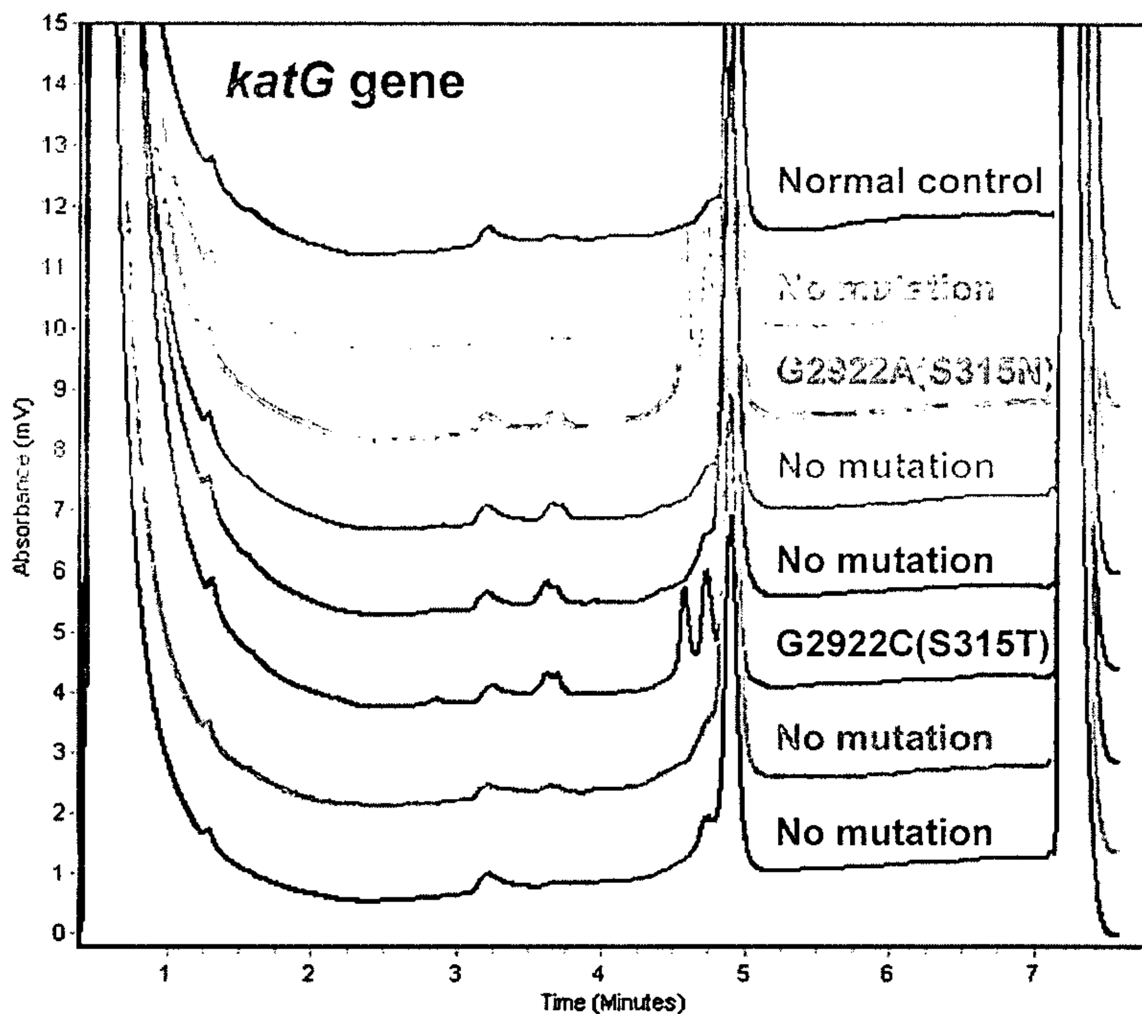

Figure 2: DHPLC detection of *rpoB* gene mutations.

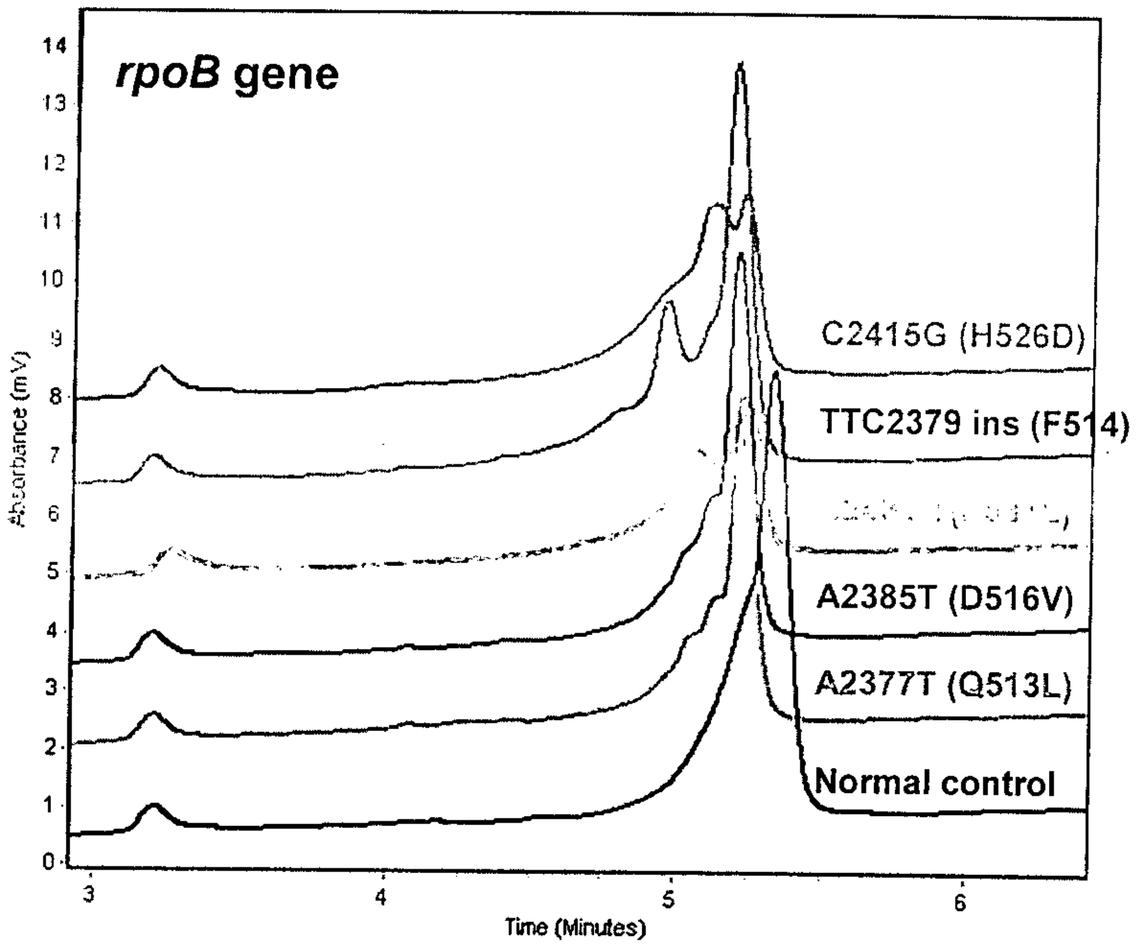

Figure 3: DHPLC detection of *rpsL* gene mutations.

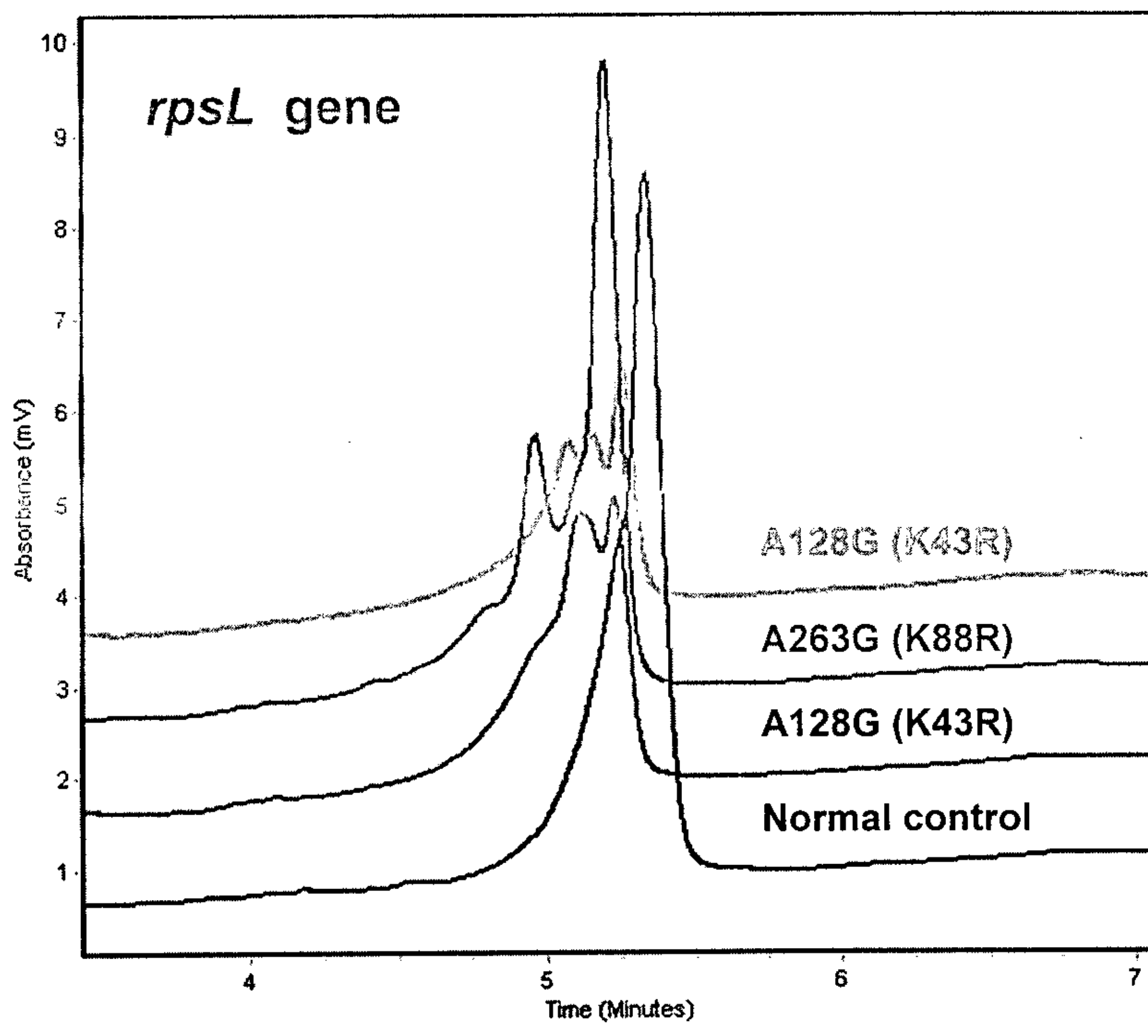

Figure 4: DHPLC detection of *embB* gene mutations.