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行政院衛生署疾病管制局九十二年度科技研究發展計畫

病原真菌快速檢驗系統之建立

- II 發展快速分子方法鑑定種別

研 究 報 告

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目　　錄

3. 計畫摘要：

（1）中文摘要：

中文關鍵詞：病原真菌、快速分子檢驗、即時聚合酶酵素連鎖反應、酵素免疫分析法、種別鑑定

近年來因人類免疫不全病毒(HIV)之感染、癌症、抗生素之濫用及器官移植、重症照護等醫療行為及人口老化等因素、導致免疫功能不全之個體大量增加，助長了病原真菌感染之趨勢。除上述機緣性感染之案例外，肇因於都市的發展、人口遷移及自然災害等因素，病原真菌對於健康個體之威脅性亦與日俱增。除此而外，新的致病原崛起、抗藥性菌株浮現，使得病原真菌逐漸成為嚴重的公衛問題，需要密切監測。

鑑於種別鑑定對於病原真菌的診療及防治的重要性，今年度本計畫之重點主要為發展快速分子種別鑑定技術。本年度針對臨床上常見之病原真菌如*Candida albicans*, *Candida glabrata*, *Candida guilliermondii*, *Candida krusei*, *Candida parapsilosis*, *Candida tropicalis* and *Cryptococcus neoformans* 共發展傳統PCR、PCR-EIA、Real-time LightCycler PCR、及Rep-PCR等快速種別檢驗方法。這些種別鑑定方法專一性高，靈敏度可達10 CFU/50μl或1pg/50μl以下。以臨床及標準菌株測試結果，與綜合germ tube , Viteck及API20C的生理生化鑑定法結果符合度達100%。若能將這些快速、高敏感、高專一性的方法納入檢驗流程並與血清、培養、鏡檢等傳統方法做整合，將能縮短並精確化檢驗流程，並有助於臨床診斷、投藥及擬定防治策略之參考。本文所發展之 PCR-EIA (48)及real-time LightCycler PCR(29)方法已發表於期刊論文。

（2）英文摘要

Keywords：pathogenic fungi, rapid molecular diagnosis, real-time polymerase chain reaction, EIA, species identification.

The advent of HIV epidemic in recent years together with the modern medical practices such as chemotherapy for cancer patients, antibiotic abuse and transplantation have resulted in an increase in the size of the population of immunocomprosed individuals. Besides, due to urban development, migration and natural disaster, fungal infections also pose threat to healthy individuals. These problems are further complicated by the emergency of new fungal pathogens and the increase of resistance to antifungals. Consequently, invasive fungal infections have become a major cause of morbidity and mortality, which has highlighted the importance of surveillance. The warm and humid climate in Taiwan is very suitable for fungus multiplication. However, most fungal pathogens are not notifiable disease and difficult to diagnose, therefore their incidence and severity were largely underestimated and neglected.

Rapid and accurate identification of the pathogenic fungi to species level is critical for disease treatment and control. The main objective of our projectfor this year is the development of rapid molecular diagnostic methods. PCR-AGE, PCR-EIA and Real-time LightCycler PCR methods were developed to identify clinically most frequently encountered fungal species, such as *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis*, *Candida tropicalis*, *Candida guilliermondii* and *Cryptococcus neoformans*. Specificty of the methods were 100%. Sensitivity were less than 10 cfu/50μl or 1pg/50μl of the fungal DNA.

The identification results matched results of the phenotypical identification method employing germ tube , Viteck and API20C methods. Therefore, the method is simple, rapid, and sensitive enough for detection and identification of several fungal species. Application of these molecular methods and integration with traditional serological, cultural and biochemical methods can shorten and streamline the fungal diagnostic processes, which will be beneficial not only for clinical diagnosis and therapy regimen but also for control of fungal diseases. The results from PCR-EIA (48) and real-time LightCycler PCR (29) methods obtained in this project have been published in peer-reviewed journals.

4. 本文

(1)前言：

過去十年來，因愛滋病毒(human immunodeficiency virus, HIV)的肆虐、癌症、抗生素及類固醇之濫用及器官及骨髓移植(81)、侵襲性療法、重症及新生兒(早產兒)照護等醫療行為的進步及人口老化等因素、導致免疫功能不全之個體存活率增加，使得真菌感染盛行率之大幅攀升(3,61)。除上述機緣性感染之案例外，肇因於都市的開發、人口遷移及自然災害等因素(74)，病原真菌對於健康個體之威脅性亦與日俱增，使得真菌感染之嚴重性漸獲重視(68)。

以美國為例，自1989年起念珠菌感染(candidiasis)已躍居院內血流性感染的第四位(66)。根據美國院內感染監視系統(National Nosocomial Infection Surveillance System, NNISS) 之傳染病死亡案例統計亦顯示，真菌性病害已由1980的1557例增至1997年的6534，排名由第十位躍升至第七位(57)。其他歐美國家之調查報告亦指向病原真菌漸趨嚴重之事實(44)。依據美國Rentz等人1998年之分析報導治療每位念珠菌血症(candidemia) 病患花費約在3萬—4萬5美元間，這是由於患者住院期長，且抗真菌用藥價昂。未來這些費用可能隨著更昂貴藥物的出現而更形增加(72)。

在國內的真菌盛行率增加情形也極類似，甚至更為嚴重。在台灣某教學醫院念珠菌則高居院內感染致病菌的榜首(12)。依據該醫院統計1981至1993年院內感染真菌由1981年的0.09%增加至1993年的0.66% 共增加了27倍，其中加護病房發生率最高(1993年為2.65%)。在菌種分析方面，前四名

分別為*Candida albicans* (50.8%)、*Candida tropicalis* (17.6%)、*Candida parapsilosis* (11.7%)及*Candida glabrata* (8.2%)。其中，*C. parapsilosis* 與 *C. glabrata* 更攀升了4-6倍的。在1994年，該院每千名出院患者中發生2.53次的念珠菌血症，佔院內血流感染的16.2%(32)。而另一教學醫院新生兒加護病房在過去的三年半內，也發生了50多次的念珠菌血症，其中甚至發生了三次院內念珠菌血流感染的流行(30,31)，在在顯示真菌感染，尤其是念珠菌感染，在今日醫療中不可輕忽的地位。除了盛行率增加快速之外，侵襲性黴菌感染所造成的合併症(morbidity)和死亡率(mortality)都相當高，死亡率一般在30-60%之間，如此高的死亡率與目前在診斷困難有相當的關係。

近年來抗真菌藥物治療真菌疾病之效果仍有許多改善的空間(5,62)。這是因為早期診斷不易，延誤投藥時機。抗真菌藥物選擇少且副作用大、對有些真菌無效及產生抗藥性等缺點。因此早期診斷以精確篩選出因罹患侵襲性黴菌感染需要接受治療的病人十分必要，此舉可望改善治療效果，減少非必要的藥物治療，節約昂貴藥物的治療費用，並避免產生副作用。此外，近來正發展多種具有不同作用範疇的替代藥物如voriconazole、posaconazole、echinocandin、caspofungin(41)，使得醫師在針對不同種別真菌用藥時有更多選擇，精確鑑定出種別，對醫師選擇藥劑種類及劑量將益形重要。

抗藥性亦已成為真菌感染的重要議題。自從azole類藥物尤其fluconazole上市以來，其低腎毒性、可口服及廣效性之優點，使其漸成為抗真菌用藥的首選之一。然而，對azole藥物具有抗藥性的菌株逐漸浮現，不同種別的真菌對抗菌藥物的抗菌程度不一樣(8)，更增加問題的棘手性

(46)。其他的藥物包括amphotericin B, flucytosine, itraconazole, ravuconazole及voriconazole對不同黴菌的抑菌濃度也有顯著不同(64)。如*Candida lusitaniae*對 amphotericin B容易產生抗藥性 (65)。對 fluconazole，*Candida krusei*完全具抗性，*C. glabrata*則較諸其他*Candida* 菌種具感受性(60)。*C. tropicalis*非常容易產生 fluconazole 的抗藥性 (4)。

病原真菌在診斷上頗為困難。早期及正確的診斷有助於病害診療及防治(17,39)。真菌種類繁多可大分為酵母菌及黴菌兩類，而會引起疾病的真菌大約在100-200種。一般較常見的致病菌包括*Candida spp*. (*C. albicans*, *C. tropicalis*, *C. parapsilosis*, and *C. glabrata*), *Cryptococcus neoformans*, *Aspergillus spp*. (*A. fumigatus*及*A. niger*等)。許多較罕見的真菌。菌種亦在近年來被發現引起真菌菌血症(fungemia)，包括*Candida inconspicua* (15), *Penicillium marneffei* (53), *Fusarium sacchari* (24), *Candida famata* (40), *Saccharomyces cerevisiae* (63), *Acremonium strictum* (80) , *Pichia anomala* (2), *Penicillium piceum* (28), *Candida lipolytica* (14), *Exophiala jeanselmei* (58), *Trichosporon asteroides* (42), *Saccharomyces boulardii*(47), *Candida dubliniensis*(56), *Trichosporon cutaneum* (10), *Cylindrocarpon* (Fusarium) *lichenicola* , *Pichia ohmeri* (75), 及*Candida versatilitis*(6)等。病原真菌之傳統鑑別診斷診斷方法一般結合培養法如用SDA、BHI、CHROMagar培養基培養及染色法如用KOH、India Ink(73)，觀察菌種的生長型態為主。然培養法費時久、過程複雜、需長時間累積的經驗。且敏感度(sensitivity)不夠高。生化生理檢測法如用API20C及API ID32C (bioMérieux Inc., France)、Vitek、Rapid檢測法等商業化快速鑑定的套組(67)，雖然操作上比較簡單，然須培養48小時才可以判斷結果，並僅能對常見菌種做鑑定，對於不常見的菌種，結果常會出現錯誤鑑定(1)。近來，一些非培養分析法陸續被發展出來包括

免疫檢測法如樹脂凝集試驗法(Latex Agglutination，LA) (33)、免疫擴散法(Immunodiffusion，ID)、酵素免疫反應(Enzyme immunoassay，EIA)(18)、免疫螢光染色 (immuno- florescence，IF)(37)、補體固定(Complement Fixation，CF)反應等方法來檢測檢體之真菌細胞壁成份、抗原、抗體及生化代謝反應(71)。例如，利用LA檢測Cryptoccocus neoformans抗原，偵測麴菌和念珠菌抗原的三明治免疫酵素法(7)，免疫螢光染色(immunoflorescence，IF)(34)、或利用EIA、ID或 CF測blastomycosis, coccidioidomycosis, paracoccidioidomycosis及histoplasmosis等之抗體力價(38)。綜言之，這些以表現型(phenotype)為主的方法都仍有若干的限制及盲點，在靈敏度及專一性上也仍有改善之空間，因此需輔助以分子生物方法以達能快速、精確及敏感偵測之目的(20,59)。晚近，核酸檢測方法如各種PCR、RFLP、RAPD、AFLP、rDNA序列分析等技術之崛起提供快速、精確及敏感偵測及鑑別之利器(39)。在疾病診斷及種別鑑定上鑑別上聚合酵素連鎖反應 (polymerase chain reaction, PCR) (9,77)最為常見。PCR具有快速鑑別pg微量或單一細胞之靈敏度。以PCR配合酵素免疫檢測法(EIA)或限制片段長度多型性(restriction fragment length polymorphism, RFLP)技術(35,79)以及核酸雜合法(25,55)檢測真菌已有不少報告。如應用在*Aspergillus spp*(23), *Candida spp.* (27,69), *Cryptococcus neoformans*(11), *Penicillium marneffei*及重要的雙型性酵母菌(51)等之鑑別。RFLP雖效果好，但需限制酶的反應一個晚上或更久的時間，實驗步驟也相當繁雜，有時還需要多個的限制酶同時並用，才可將菌株明確的區分。針對檢驗病原真菌中*Coccidiodes immites*、*Histoplasma capsulatum*及*Blastomyces dermatitidis*的標準化核酸檢驗試劑(GenProbe)已成功上市。PCR的最新發展則是利用及時PCR偵測法(real-time PCR assay)，及時PCR偵測法具有快速(45min-2hr)、敏感度更高、可定量、可避免污染等優點。用來檢測*Pneumocystis carinii*肺炎

(45)、*C. albicans*、*C. neoformans*等病原。Repetitive PCR (rep-PCR)或IR-PCR分析則是利用菌株重複序列多複本、多型性及變異性之特點，設計引子與欲鑑別樣品進行聚合酵素連鎖反應，產生高度多型性PCR產物(70)。

近來自動化定序技術越臻成熟，成本越趨合理，使得定序用於鑑定工作可行性增高。有許多研究者也有以菌種之間序列的差異來鑑定菌種(13,26)。最常見是用普遍性引子擴增真菌之rDNA片段，然後以PCR產物之序列不同來辨別菌種。Sugita等已經建立一個ITS序列資料庫來鑑定臨床重要的*Trichosporon* species，並且只需要一個工作天或24小時的鑑定時間(78)。Ninet等發展一套利用28S核酸基因序列來鑑定皮膚真菌菌種(dermatophytes species)的商業套組，能將9種重要的致病菌鑑定出來。ABI公司則發展出D2 rDNA Fungal Identification System針對真菌的D2 LSU rDNA片段可全自動化分析序列，並經由資料庫比對鑑別種別。然而真菌因基因體較大，多數為雙倍體，有關標定基因的選擇仍未有定論等問題仍有待克服。這些資料若能更臻完備，不但可作為分類鑑定之依據，更可提供引子、探針設計的寶貴資料。將來，希望這些序列資料庫能儲存在疾病管制局基因資料庫平台，供國內外研究學者共享。

要言之，因致病性真菌引發的疾病其嚴重性及致死率逐年增加、新的致病原崛起(68)、抗藥性菌株浮現，使得病原真菌逐漸成為嚴重的公衛問題，需要公共衛生單位密切地監測。希望藉由本研究發展之快速分子診斷技術，對於病原真菌的快速診斷能有所貢獻。

(2)材料與方法：

a. 菌株來源及培養:

Candida albicans (ATCC14053), *Candida glabrata* (ATCC2001), *Candida krusei* (ATCC6258), *Candida parapsilosis* (ATCC20515), *Candida parapsilosis* (ATCC22019), *Candida tropicalis* (ATCC750) 標準菌株由台大醫院陳宜君醫師提供；*Candida guilliermondii* (BCRC21559), *Cryptococcus neoformans*（BCRC22873）標準菌株由成大醫技系張長泉教授提供，臨床菌株來自國家衛生研究院羅秀容副研究員民國 88 年 4 月 15 日至 6 月 15 日從全省 22 家醫院收集之臨床菌株 (台灣酵母菌抗藥性第一期監測計畫，Taiwan Surveillance of Antimicrobial Resistance of Yeasts I, TSARY I 計畫) (52)。PCR-電泳凝膠及 PCR-EIA 檢測法所用標準及臨床菌株列於表一。Real-timeLightCycler PCR 檢測法所用標準及臨床菌株列於表二。菌株用 Sabouraud dextrose agar (SDA)培養於 37℃,72 小時。

b. 菌株之型態及生理生化鑑定：

先採用發芽管試驗(Germ-tube assay)，接著採用常用之 API-32 或 VITEK 酵母菌生化卡片自動鑑定系統。

c. DNA 萃取：

真菌分離株培養後用 PUREGENE DNA Purification Kit (Gentra, Minneapolis, Minnesota, USA)萃取 DNA。簡言之，在 Sabouraud dextrose agar (SDA)上培養 2~3 天後，取兩個接種環大小的真菌量攪散在裝有 2ml PBS 的 5-ml 滅菌試管內，加入 10-15 μl 分解酵素(lyticase)，置於 37℃過

夜。菌液以 13,000 x g 離心 3 分鐘之後，倒掉上清液；加入 2 ml Cell Lysis Solution，將細胞胚累沖散以達到分解細胞的效果。之後加入 1 ml 蛋白質沈澱液，以 vortex 最高速震盪 20 秒；接著以 13,000 x g 離心 10 分鐘。取上清液加入 100%異丙醇使 DNA 沉澱；以 70%酒精洗過後，風乾再以 50 μl of DNA Hydration Solution 讓 DNA 溶水。接著抽出 DNA 的以光度比色計用 *A260* 波長測量濃度。DNA 冰存於 -80℃待用。

d. 瓊脂膠體電泳分析（agarose gel electrophoresis）：

使用 2.0% (wt/vol)的瓊脂膠體 (BioWhittaker Molecular Applications: BMA, Rockland, ME, USA)搭配 1X 的 TBE 緩衝溶液(0.1 M Tris, 0.09 M boric acid, 1 mM EDTA [pH 8.4]) 100 伏特進行電泳 1~2 小時；每個待測樣本取 5 μl 並以 100-bp DNA 分子量標準片段同時跑做對照。電泳結束後，於每毫升 0.5 μg ethidium bromide (EtBr) 染劑中染 15 分鐘，接者以蒸餾水去染數次，每次 30 分鐘。

e. PCR引子之設計：

參考文獻(19,50,76)並從NCBI等基因序列公共資料庫蒐尋相關序列資料，加以排序比對後（圖一），設計各真菌之引子及核酸探針。

- PCR-AGE：使用針對ITS2的種別引子，詳細序列見表三。
- PCR-EIA： 使用之泛真菌ITS1及ITS4及針對ITS2的種別引子/探針。種別引子標誌有 digoxigenin，ITS3 序列在5′端標誌有biotin。詳細序列見表四。引子及探針所針對之rRNA部位詳見圖二。
- Real-time PCR之引子及探針設計使用Light-Cycler儀器所附軟體。種別專一性引子(CALB, CGL, CPA, CTR, CGU, CKRU and CN)主要是

針對rDNA的 ITS1 及 ITS2 區域，其位置及序列如圖三及表五(49,54)所描述。

f. PCR增幅：

增幅引子使用針對rDNA的泛真菌(pan-fungal)或種別專一性引子。PCR反應容積為50 μl，內含1 μl待測DNA，25 μl 2 X PCR緩衝液（MBI Fermentas 2X PCR Master Mix），0.5 μM各種引子，其餘加蒸餾水混勻。增幅初始變性反應94℃ 5分鐘溫度，35次循環的變性反應94℃ 30秒→黏和58℃ 30秒→72℃ 1分鐘聚合延長反應，最後為72℃ 10分鐘聚合延長反應。PCR機器使用PTC-200(MJ research)。試劑製備反應全程均有依據Kwok 及 Higuchi 的預防污染建議(43)。

g. EIA：

將泛真菌引子增幅之片段取 10 μl 先加熱 95℃,5 分鐘，接著置於冰上，加入 200 μl 的 hybridization buffer (4× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], pH 7.0, 0.02 M HEPES, 0.002 M EDTA, and 0.15% Tween 20)及 10ng 的 ITS3-標誌有 biotin 的泛真菌探針和 10ng 的標誌 digoxigenin 的種別探針。混合後置於 37℃、1 小時。將 100 μl 混合液加入事先黏附有 strepavidin 的 96 孔盤，於室溫下震盪(~350 rpm)培養 1 小時後以 0.01M PBS- Tween 洗六次。接著加入 100 μl 的 1：1000 倍稀釋的 horseradish peroxidase 標定的抗 dioxigenin 抗體(150 U/ml; Roche)，於室溫下震盪培養 1 小時後以 0.01M PBS-Tween 洗六次。最後加入 3,3', 5,5'-Tetra- methylbenzidine (TMB)-H_2O_2 基質，反應 10 分鐘後，於光度計(Quant Universal Microplate Spectrophotometer，BIO-TEK

INSTRUMENTS, INC. USA)以 650nm 讀取吸光值（圖四）。

測試敏感度，利用純粹培養之 *C. albicans* 調成 3.0 MacFarland 單位 (約為 $1x10^6$ CFU/ml)，接著直接將菌液以 100℃煮 5 分鐘。將煮過的懸浮液做十倍系列稀釋，當做 PCR-AGE 或 PCR-EIA 的 template DNA。或抽取 DNA，將抽取的 DNA 從 1ng 到 1fg 依序做 10 倍序列稀釋。

h. Real-time LightCycler PCR:

使用儀器為Light Cycler P CR增幅及偵測系統 (Roche Diagnostics, Mannheim, Germany)。PCR反應在封閉之毛細管中進行，由於毛細管之比表面積高，且循環反應則經由交替加熱氣體達成，故可快速達到反應物與氣體的平衡。增幅產物之偵測則使用 Light Cycler FastStart DNA Master SYBR Green 螢光試劑套組。SYBR Green 1螢光染劑會嵌合雙股DNA，使用依照廠商說明書。PCR反應混合物 (20μl)包括 *Taq* polymerase, 1× Light Cycler reaction buffer, 3mM magnesium chloride及 0.5 μM 的primers。加入1ng待測DNA。反應條件為35 次循環的變性 (5秒 95 ℃), 黏合 (5秒 58 ℃)及酵素延長反應 (25秒 72 ℃)。接著從60°C升溫至95 °C以分析增幅子融化曲線(melting curve)，先接著降溫至 40 °C。整個PCR反應在45分鐘結束。PCR的過程可由螢幕上即時監控SYBR Green 1 DNA結合染劑螢光值增加來定量。

進行 real-time PCR 敏感度之測試。將抽出之 DNA 序列稀釋成不同濃度，當作 template DNA 以界定其偵測極限。

(3)結果：

利用 PCR-電泳凝膠（PCR—Agarose gel electrophoresis）快速檢定方法以 ITS3 及種別專一性引子(CAL, CGL, CPA, CKR, CTR,CGU,CN)針對各種病原真菌做 PCR。可鑑別包含 *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. Krusei*, *C. tropicalis*, *C. guilliermondii* and *C. neoformans* , 7 種臨床上重要真菌，專一性高達 100％，靈敏度高達 10 CFU，所需時間< 2.5h。產生增幅子片段約為 200～300bp (圖五)。ITS3 及 CAL 引子可將 *C. albicans* 增幅出一約 260-bp 片段產物，與其他六種 *Candida* spp. 的 DNA 均無反應。 ITS3 及 CAP 引子與 *C. parapsilosis* 可增幅出約 250-bp 的產物與其他六種 *Candida* spp.的 DNA 均無反應(圖六), 其他種別引子亦僅針對同種的 DNA 會有 PCR 反應，他種的則沒有反應(圖未列出)。將 *C. albicans* 調成 10^6 至 10^0 cells /ml 不同菌液濃度以 primer ITS3 和 CAL 進行 PCR 反應並測試其敏感度，結果顯示菌液濃度稀釋至 10^4 cells /m1（l0 CFU 每 50μl 反應容積）仍可以見到約 270bp 之產物（圖七）。

PCR-EIA 以標準菌株做試驗顯示，鑑別 7 種臨床上重要真菌，七種真菌均有反應，且這些探針只與同種病原的 DNA 有雜和反應，與他種 DNA 則無雜和呈色反應，專一性高達 100％（表六），種別特異性探針偵測病原真菌之敏感度為 10 CFU /50 μl（表七）或 1pg/50 μl（表八）。所需時間<4h，可定量，可鑑別混合感染，具有大規模處理及自動化之潛力。以 51 株臨床菌株包含 *C. albicans* (n=16), *C. glabrata* (n=8), *C. parapsilosis* (n=6), *C. Krusei* (n=4), *C. tropicalis* (n=9), *C. guilliermondii* (n=7) and *C. neoformans* (n=2)分別測試 PCR-AGE 及 PCR-EIA 鑑定結果。以 PCR-AGE 或 PCR-EIA 鑑定，51 株鑑定結果均吻合生化檢驗結果。

Real-time-PCR 快速種別鑑定方法可鑑別 7 種臨床上重要真菌，專一性高達 100%，靈敏度高達 10fg，所需時間<1h，可定量，可鑑別混合感染。Real-time LightCycler PCR 快速檢定方法增幅 rDNA 區段，進一步分析溶解溫度(melting temperature, Tm)鑑定臨床重要酵母菌。為測試 LightCycler 的專一性，以 C. albicans ATCC14053 萃取的 DNA 測試專一性引子 CALB1 和 CALB2 和 *C. albicans* 的黏合增幅。偵測極限約為 1 pg/µl (圖七)。其他種別的 DNA 也獲得類似的結果，亦即每個種別僅與該種的專一性引子作用，與其他種別則無反應。將 *C. albicans* ATCC 14053 純化的 DNA 做 10 倍序列稀釋以測試線性範圍，並將螢光值對應循環數做曲線。線性達 4 個 log，範圍從 1 ng 至 1 pg/µl 濃度的真菌 DNA (圖八)。電泳凝膠圖顯示參考菌株的從 1 ng to 1 pg/µl DNA 序列稀釋樣本與其對應專一性引子作用後均僅有單一 band (圖九)。

相較於細菌菌血症與病毒菌血症，真菌菌血症之菌量一般低很多。因此，高敏感度的偵測對於疾病及時的治療甚為重要。將六個 *Candida* species 及一個 *C. neoformans* 的 DNA 用各別的種別專一性引子於 LightCycler 增幅以評估檢測方法的敏感度，結果顯示專一性達 100%，敏感度極限約為 1 pg/µl(圖十～圖二十一)。

測定臨床菌株以決定LightCycler 方法應用於種別鑑定的正確性。分析58株臨床菌株的結果顯示，不論臨床菌株的分離部位或地理區來源相同與否，real-time PCR方法的鑑定結果與生化檢測法完全相符。種別專一性引子與其對應種別真菌DNA作用可得到特定增幅子，分析增幅子的溶解曲線可得到獨特的Tm值高峰(表九、表十)。

(4) 討論

真菌性病害近年來的顯著增加及其高死亡率，凸顯了真菌致病原快速及正確鑑別診斷的重要性。隨著抗真菌藥物發展的進展，提供了不同種別及抗藥性投藥的多種選擇，使得正確種別鑑定更形重要。

本計畫共建立 PCR-AGE、PCR-EIA 及 real-time PCR 三種所建立快速檢定方法。其中 PCR-AGE 屬於較傳統普遍的方法，成本最低。PCR-AGE 可鑑別 7 種臨床上重要真菌，專一性高達 100%，靈敏度高達 10 CFU，所需時間<2.5h。

PCR-EIA 快速檢定方法，可鑑別 7 種臨床上重要真菌，專一性高達 100%，靈敏度高達 10 CFU/ml，所需時間小於 4 小時。*C. krusei* 由於增幅片段的 G+C 比例偏高，原先無法被鑑定出來，後來可經由改善增幅子變性條件及增長雜合反應的條件加以改善。以 51 株臨床及 7 株標準菌株測試結果，與綜合 germ tube，Viteck 及 API20C 的生理生化鑑定法結果符合度達 100%。使用的 DNA 模版可使用萃取的 DNA 也可以直接用全菌體。直接用全菌體更可簡化步驟，降低污染的風險。本方法具有可自動化及大量檢體處理之潛力，此外，二種以上的混合感染也能被偵測出來。

Light Cycler real-time PCR 技術需時最短(45 分鐘)。可提供快速、高敏感度、低污染機率、精確種別鑑定之選擇。利用種別性專一引子，僅與該引子同種的病原 DNA 會被增幅出來，不同種別的 DNA 則無反應。增幅標的在 rDNA 區段，病原真菌種別鑑定的標的包括 18S 核醣體 DNA (rDNA)基因，粒腺體 DNA (82)，轉錄區間內(Internal Transcribed

Spacer; ITS) 序列，及其他基因(36)。使用 rDNA 基因當標的基因的優點為，由於具有約 100 個複本故可大幅度提高敏感度。分析 ITS1 (Chen et al., 2001)或 ITS2 (16)片段長度變化亦有學者報導可用來區分種別。使用所發展的 LightCycler 系統提供另一優點為可進一步分析溶解溫度(melting temperature, Tm)輔助鑑定。Tm 值受核甘酸 G+C 含量，片段長度及核甘酸組合變化的影響。每一種別特異性片段有其特定 Tm 值，這有助於進一步確認病原真菌之種別。

病原真菌基因序列之相繼解碼,提供了分子生物診斷技術莫大的契機。經由序列的搜尋比對，可依實驗需要設計出鑑別不同種之病原真菌的專一性引子及探針。本實驗使用的序列區域屬真菌核醣體DNA基因或間隔轉錄區(ITS)序列。本計畫針對台灣重要病原真菌如*Cryptococcus neoformans*、*Candida spp.*…等發展出PCR-AGE、PCR-EIA及real-time PCR快速分子診斷法，專一性相當高，且靈敏度可達10cfu (PCR-AGE, PCR-EIA)或1 pg(real-time PCR)。所發展出之PCR偵測法與血清、培養、鏡檢等傳統方法做整合，有助於快速兼具高準確性之檢驗操作流程之建立。

(5) 結論與建議

真菌性病害近年來的顯著增加及其高死亡率，凸顯了真菌致病原防治的重要性。隨著抗真菌藥物發展的進展，提供了不同種別及抗藥性投藥的多種選擇，使得正確種別鑑定更形重要。快速正確之種別鑑定不僅有助於疾病的治療更可有助於抗真菌藥物合理的使用。傳統的鑑定方法主要依賴型態及生化反應等表現型。這些方法不僅耗時久且易流於主觀。因此本文描述發展 PCR-EIA 及 LightCycler PCR 快速檢定方法，鑑別 *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, *Candida guilliermondii* 及 *Crytococcus neoformans* 7 種臨床上重要真菌。

要言之，本文所發展之 PCR-EIA (48)及 real-time LightCycler PCR(29)方法簡單、快速兼具有高專一性及敏感度，提供了傳統鑑定方法以外的選擇，real-time PCR 測試法更結合了快速增幅 DNA 同時及時鑑定種別。傳統例行性 block cycler PCR 方法能很方便地轉移至 real-time 方法。若能與血清、培養、鏡檢等傳統方法做整合，有助於增加檢驗之準確性及效率，使得檢驗實驗室之工作流程更形精簡流暢。

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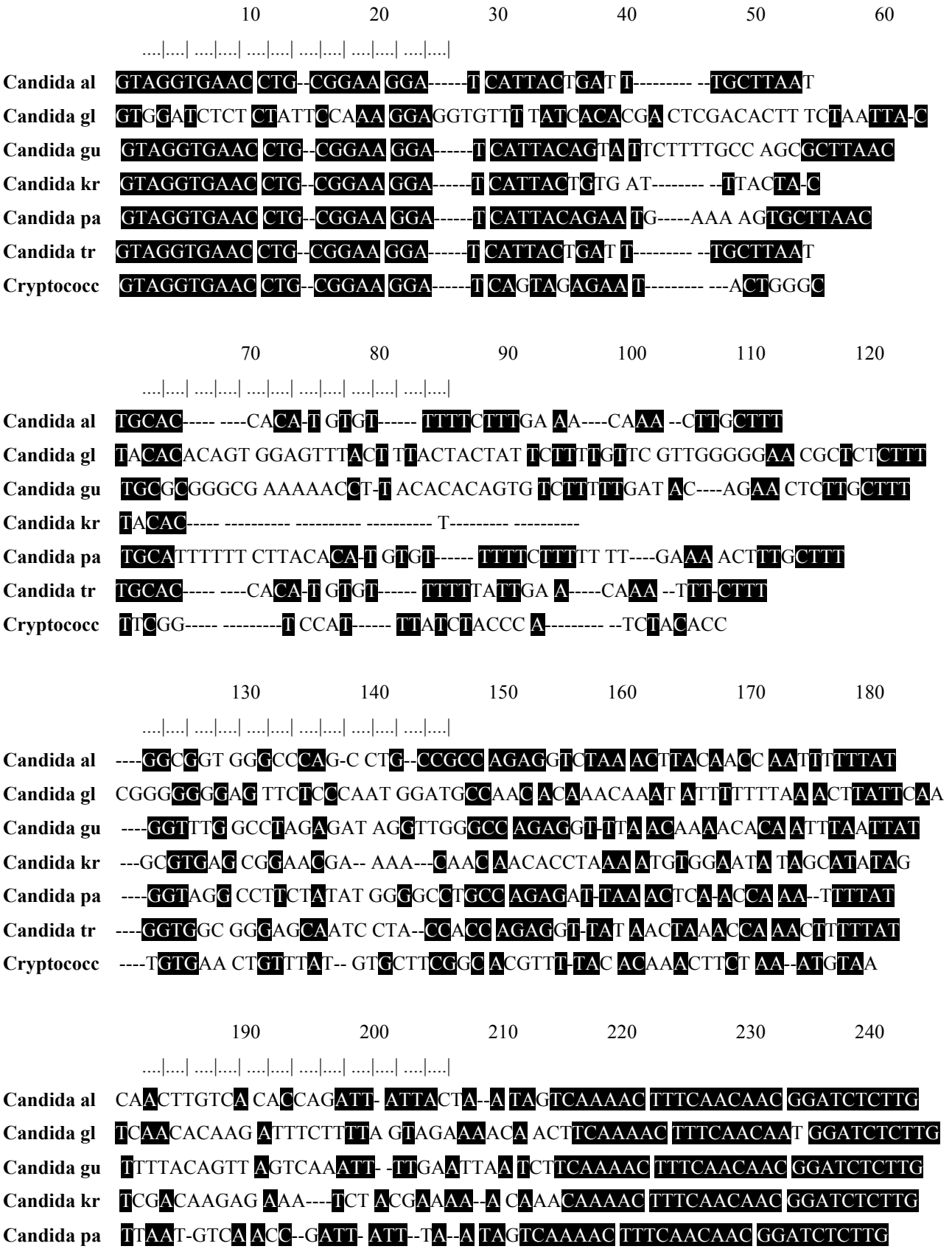
(7) 圖、表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Candida kr GTAGGTGAAC CTG--CGGAA GGA------T CATTAC TGTG AT-------- --TTACTA-C
Candida pa GTAGGTGAAC CTG--CGGAA GGA------T CATTACAGAA TG-----AAA AGTGCTTAAC
Candida tr GTAGGTGAAC CTG--CGGAA GGA------T CATTAC TGAT T--------- --TGCTTAAT
Cryptococc GTAGGTGAAC CTG--CGGAA GGA------T CA GTA GAGAA T--------- ---ACTGGGC
70 80 90 100 110 120
Candida al TGCAC----- ----CACA-T GTGT------ TTTTCTTTGA AA----CAAA --CTTGCTTT
Candida gl TACACACAGT GGAGTTTACT TTACTACTAT TCTTTTGTTC GTTGGGGGAA CGCTCTCTTT
Candida gu TGCGCGGGCG AAAAACCT-T ACACACAGTG TCTTTTTGAT AC----AGAA CTCTTGCTTT
Candida kr TACAC----- ---------- ---------- ---------- T--------- ----------
Candida pa TGCATTTTTT CTTACACA-T GTGT------ TTTTCTTTTT TT----GAAA ACTTTGCTTT
Candida tr TGCAC----- ----CACA-T GTGT------ TTTTTATTGA A-----CAAA --TTT-CTTT
Cryptococc TTCGG----- ---------T CCAT------ TTATCTACCC A--------- --TCTACACC
130 140 150 160 170 180
Candida al ----GGCGGT GGGCCCAG-C CTG--CCGCC AGAGGTCTAA ACTTACAACC AATTTTTTAT
Candida gl CGGGGGGGAG TTCTCCCAAT GGATGCCAAC ACAAACAAAT ATTTTTTTAA ACTTATTCAA
Candida gu ----GGTTTG GCCTAGAGAT AGGTTGGGCC AGAGGT-TTA ACAAAACACA ATTTAATTAT
Candida kr ---GCGTGAG CGGAACGA-- AAA---CAAC AACACCTAAA ATGTGGAATA TAGCATATAG
Candida pa ----GGTAGG CCTTCTATAT GGGGGCTGCC AGAGAT-TAA ACTCA-ACCA AA--TTTTAT
Candida tr ----GGTGGC GGGAGCAATC CTA--CCACC AGAGGT-TAT AACTAAACCA AACTTTTTAT
Cryptococc ----TGTGAA CTGTTTAT-- GTGCTTCGGC ACGTTT-TAC ACAAACTTCT AA--ATGTAA
190 200 210 220 230 240
Candida al CAACTTGTCA CACCAGATT- ATTACTA--A TAGTCAAAAC TTTCAACAAC GGATCTCTTG
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Candida kr TCGACAAGAG AAA----TCT ACGAAAA--A CAAACAAAAC TTTCAACAAC GGATCTCTTG
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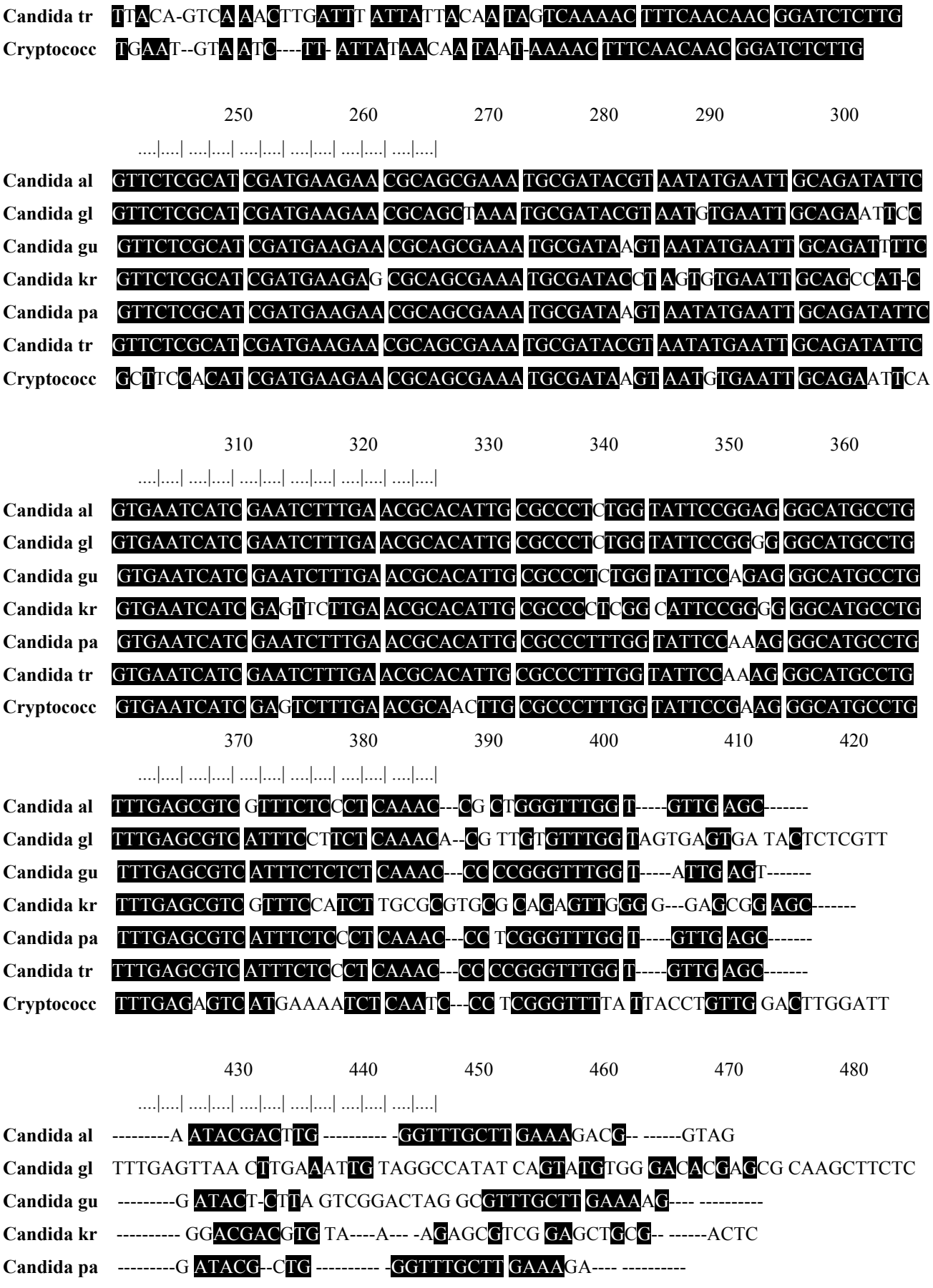
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250 260 270 280 290 300
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Candida pa GTGAATCATC GAATCTTTGA ACGCACATTG CGCCCTTTGG TATTCCAAAG GGCATGCCTG
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Cryptococc GTGAATCATC GAGTCTTTGA ACGCAACTTG CGCCCTTTGG TATTCCGAAG GGCATGCCTG
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Candida gu TTTGAGCGTC ATTTCTCTCT CAAAC---CC CCGGGTTTGG T-----ATTG AGT-------
Candida kr TTTGAGCGTC GTTTCCATCT TGCGCGTGCG CAGAGTTGGG G---GAGCGG AGC-------
Candida pa TTTGAGCGTC ATTTCTCCCT CAAAC---CC TCGGGTTTGG T-----GTTG AGC-------
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Cryptococc TTTGAGAGTC ATGAAAATCT CAATC---CC TCGGGTTTTA TTACCTGTTG GACTTGGATT
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Candida gu ----------G ATACT-CTTA GTCGGACTAG GCGTTTGCTT GAAAAG---- ----------
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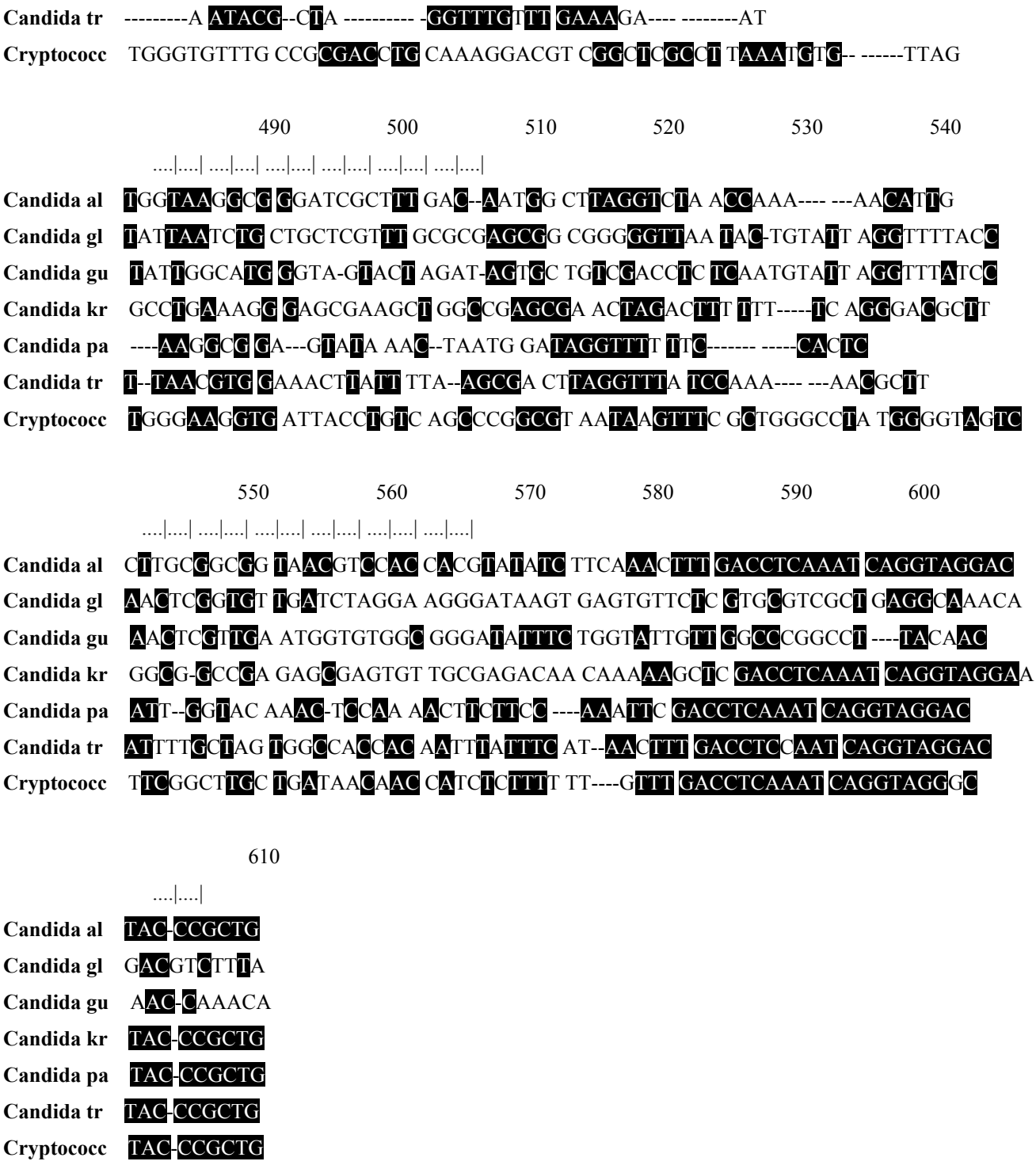
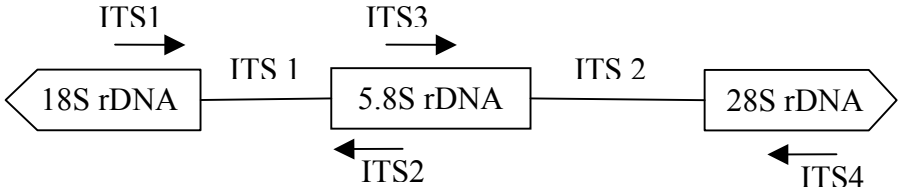
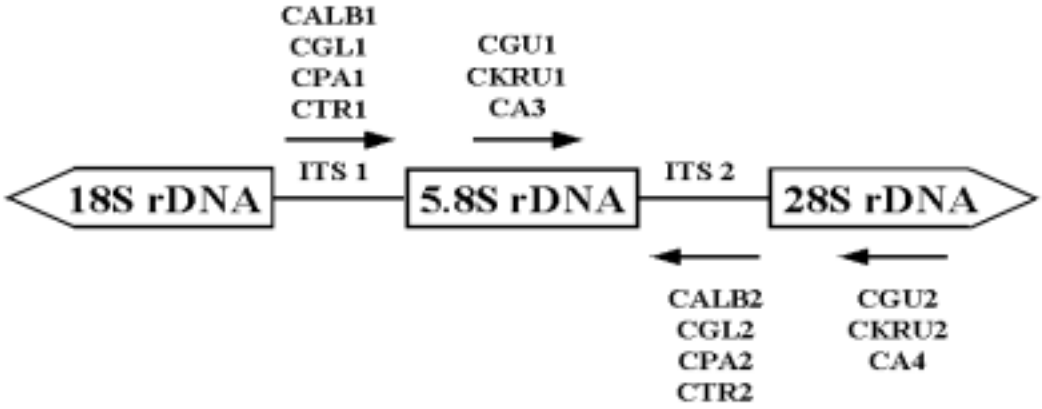


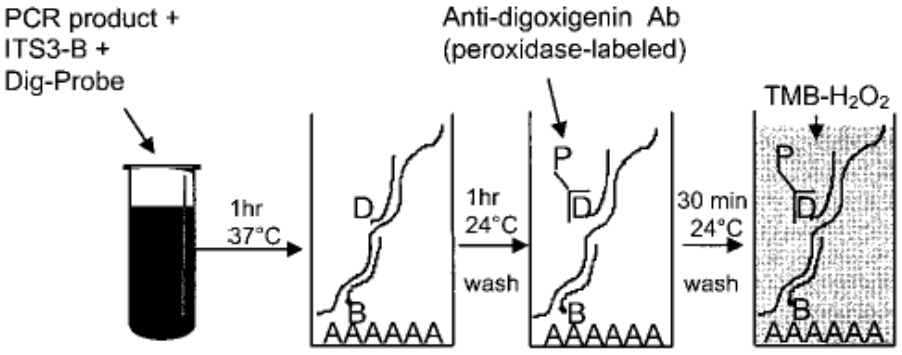
圖一、從 NCBI 序列資料庫所搜尋七種病原真菌 ITS1-ITS4 序列之比對



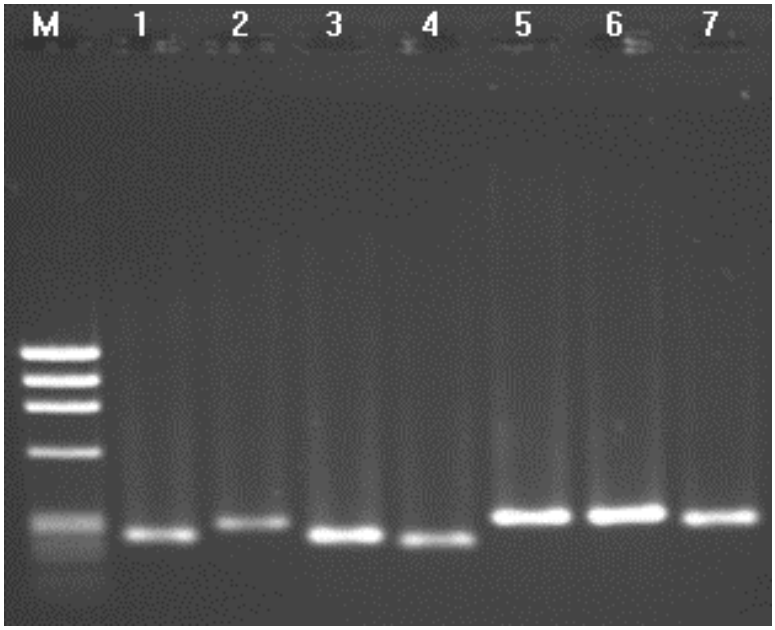
圖二、 PCR-EIA所用泛真菌引子(pan-fungal primers) ITS1-ITS4及種別專一性引子在真菌rDNA之區域。



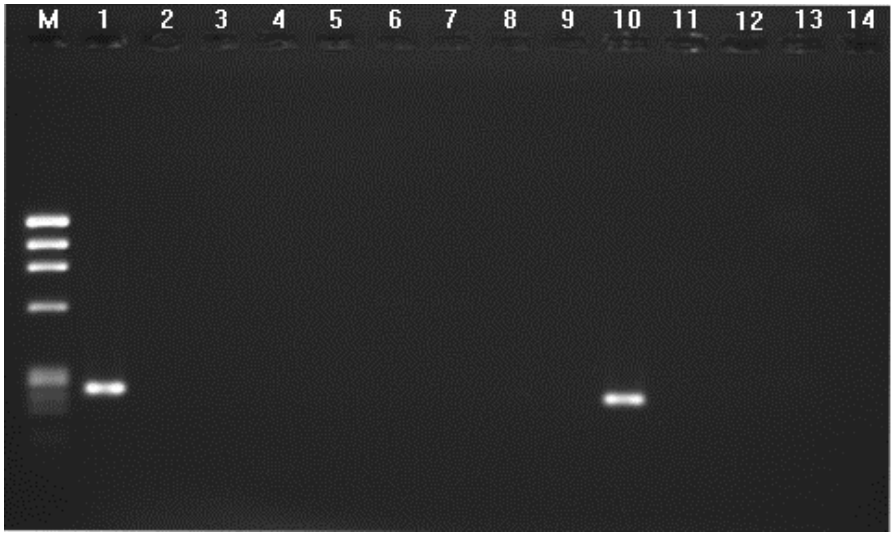
圖三、Real-time PCR所使用之種別專一性引子



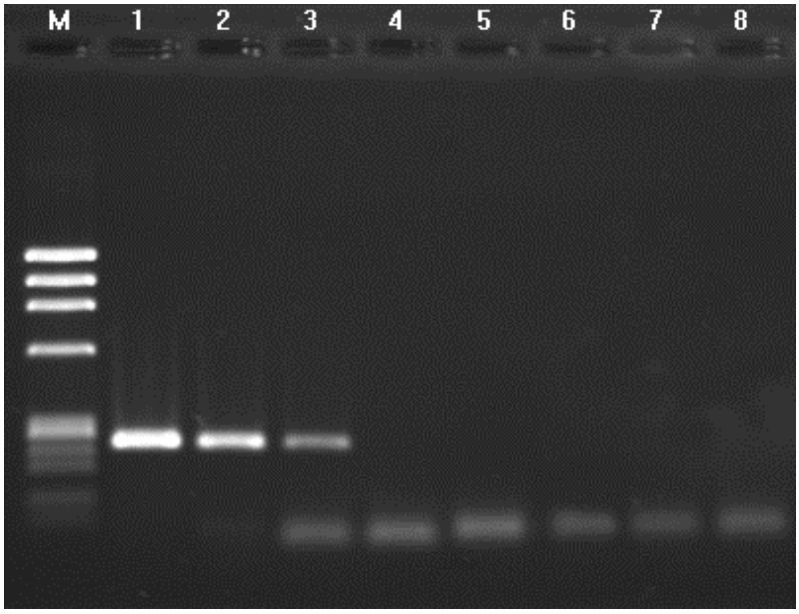
圖四、PCR—EIA步驟流程圖。PCR 增幅產物加熱使雙股變性後，先後與biotinylated 泛真菌探針 (ITS3-B) 及digoxigenin-標定的種別專一性探針(Dig-Probe) ，接著加入黏附有streptavidin (AAAAAA)的96孔盤。產物與探針是否雜合成功可經由peroxidase酵素標定 (P)的抗digoxigenin抗體及其hydrogen peroxide (TMB-H2O2) 受質(substrate)的呈色反應得知(51)。



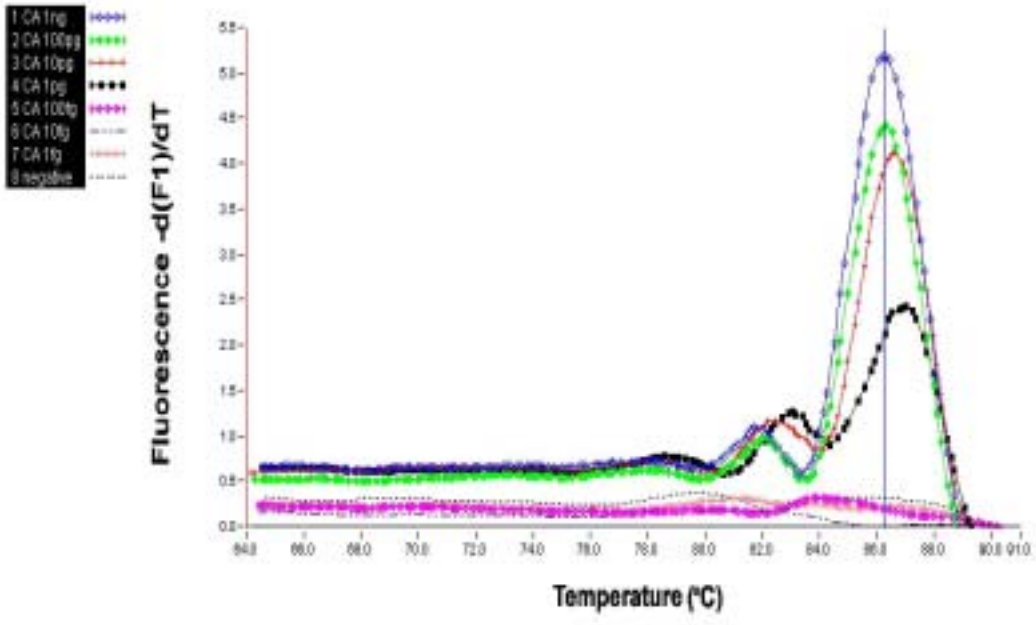
圖五、利用種別專一性引子針對真菌DNA做PCR。Lanes M：分子量標記(*Hae*III-digested X174 replicative-form DNA); Lanes 1至7分別為PCR產物*C. albicans*, primer CAL; *C. glabrata* , primer CGL, *C. parapsilosis*, primer CPA; *C. kruseis*, primer CKR; *C. tropicalis*, primer CKR; *C. guilliermondii*,primer CGU; *C. neoformans* primer CN。



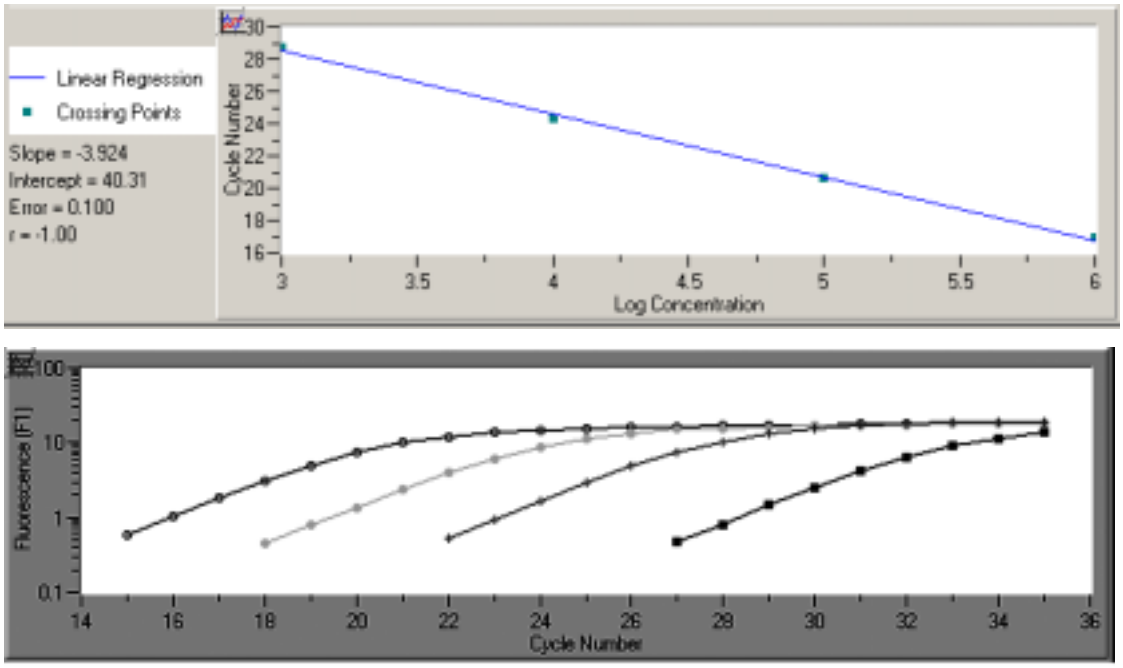
圖六 以PCR-電泳凝膠分析測試引子的專一性。Lanes 1 到 7 使用CAL 引子對分別增幅 *C. albicans*, *C. glabrata*, *C. parapsilosis* , *C. kursei* , *C. tropicalis* , *C. guilliermondii* , *C. neoformans* 標準菌株的DNA。 Lanes 8 到 14 使用 CAP 引子對分別增幅 *C. albicans*, *C. glabrata*, *C. parapsilosis* , *C. kursei* , *C. tropicalis* , *C. guilliermondii* , *C. neoformans* 標準菌株的DNA。Lanes M, 分子量標記。



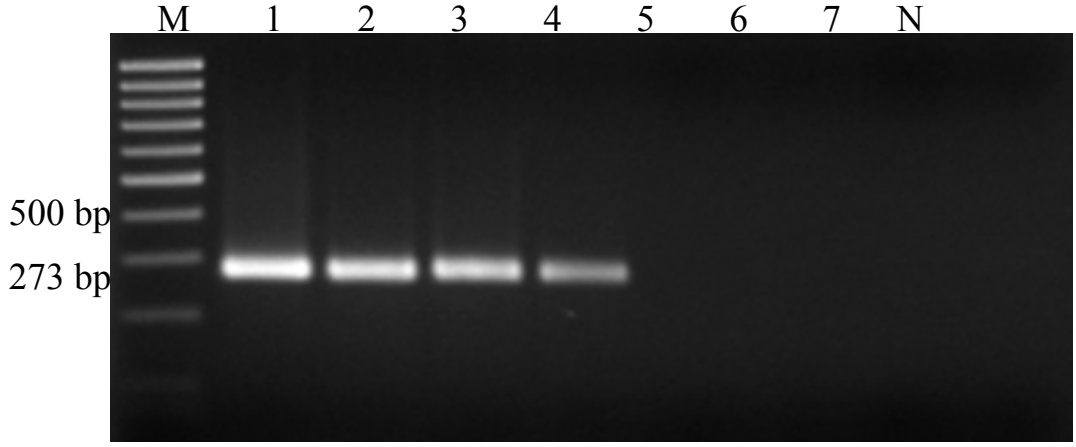
圖七、以*C. albicans* (ATCC14053)調成不同菌液濃度測試PCR之敏感度，primer ITS3與CAL測試： lanes 1 到 8 分別為每毫升 $1x10^6$, $1x10^5$, $1x10^4$, $1x10^3$, $1x10^2$, 10, 1 CFU, Lanes M, 分子量標記。



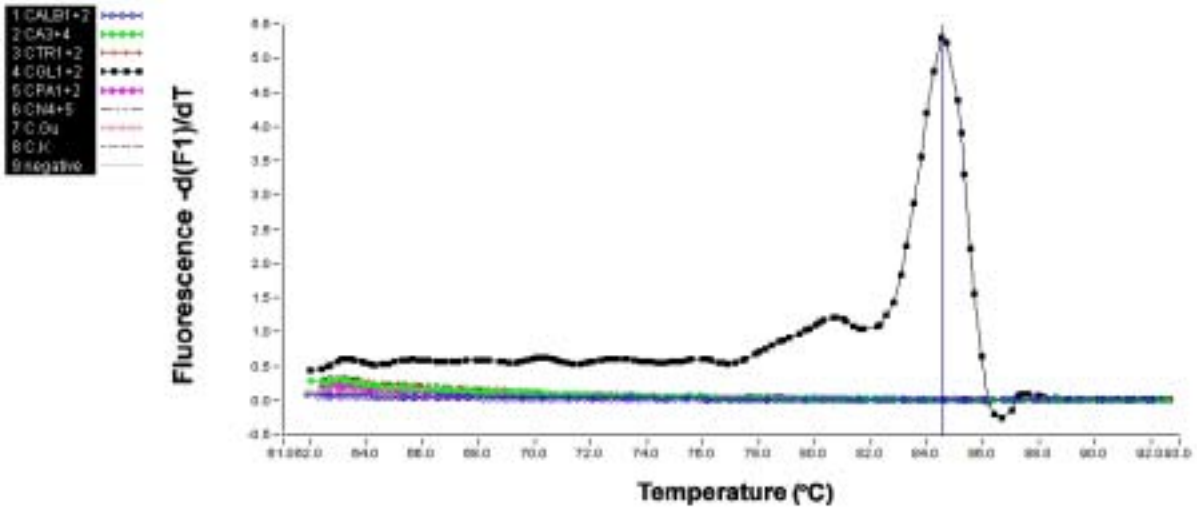
圖八、使用 CALB1 及 CALB2 引子對以 real-time PCR 分析 *C. albicans* (ATCC14053) DNA 的專一性及敏感度。可偵測 *C. albicans* 之 DNA 濃度為 1ng~100fg 。使用其他引子對(CGL、CGU、CKRU、CPA、CTR、CN)則無反應引子。



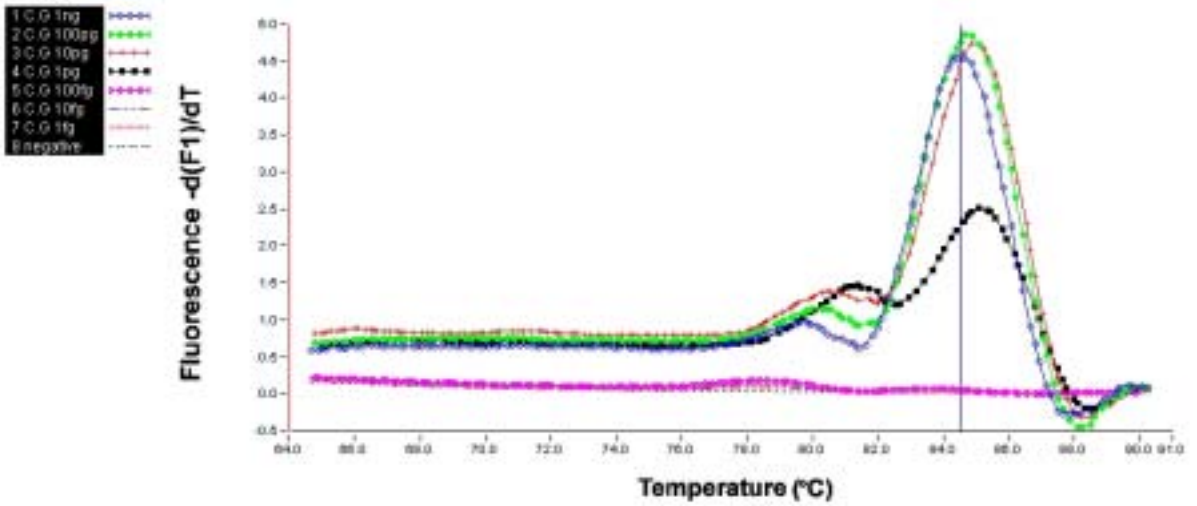
圖九、檢測*C. albicans ATCC14053*敏感度標準曲線圖(1ng~1pg/µl). 將濃度的log值(per µl) 對螢光作圖。



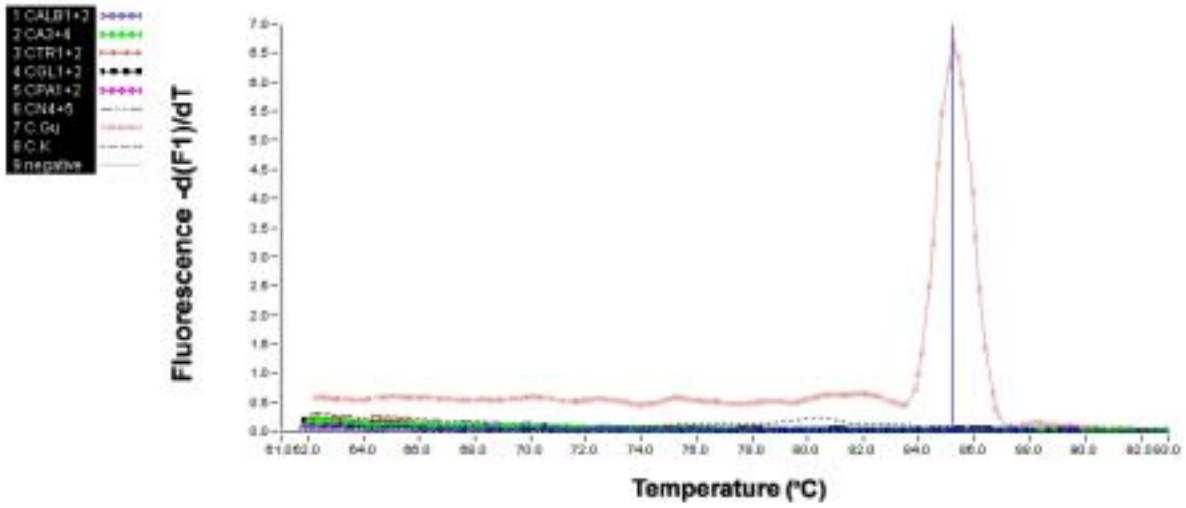
圖十、電泳凝膠圖顯示LightCycler 增幅*C. albicans* ATCC14053的DNA片段 顯示單一275 bp的專一性片段。Lines: M; marker; 1, 1ng/µl ; 2, 100pg/µl; 3, 10pg/µl; 4, 1pg/µl; 5, 100fg/µl; 6, 10fg/µl; N, negative control (distilled H2O)。



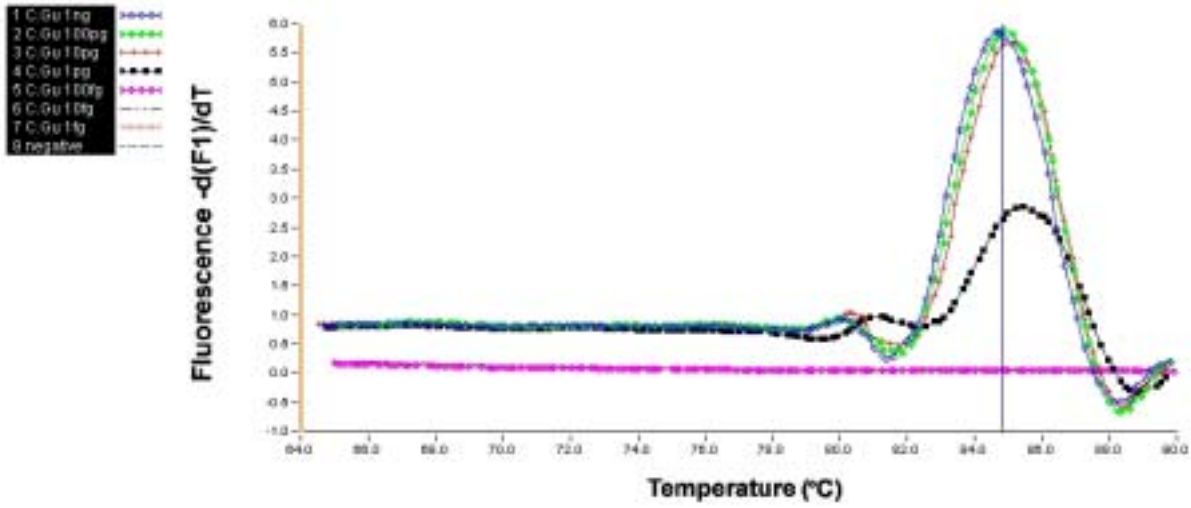
圖十一、使用CGL1及CGL2引子對以real-time PCR分析*C. glabrata* (ATCC2001) DNA的專一性。



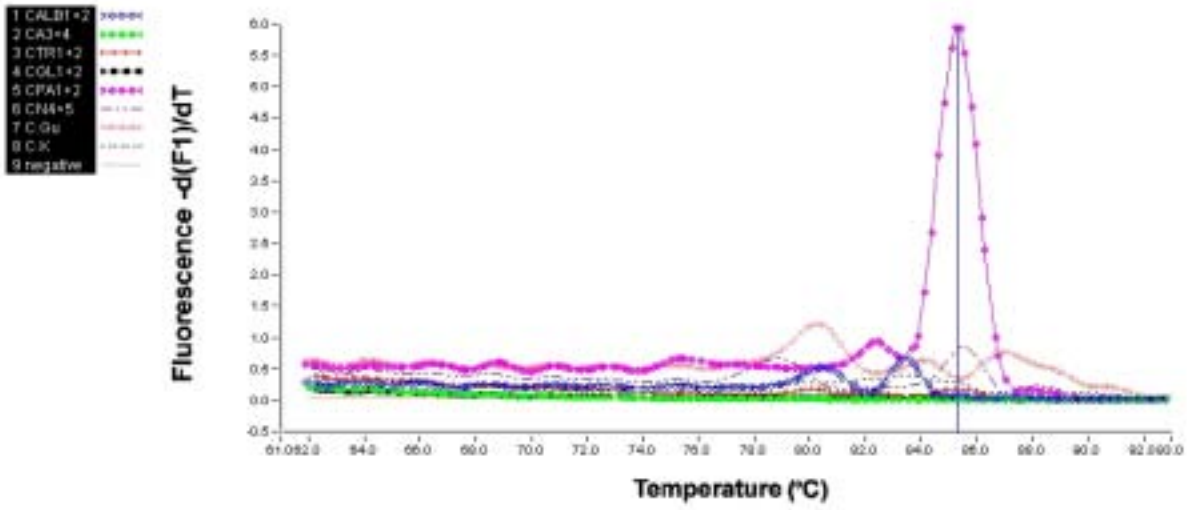
圖十二、使用CGL1及CGL2引子對以real-time PCR分析*C. glabrata* (ATCC2001) DNA的敏感度。可偵測*C. glabrata*之DNA濃度為1ng~100fg。



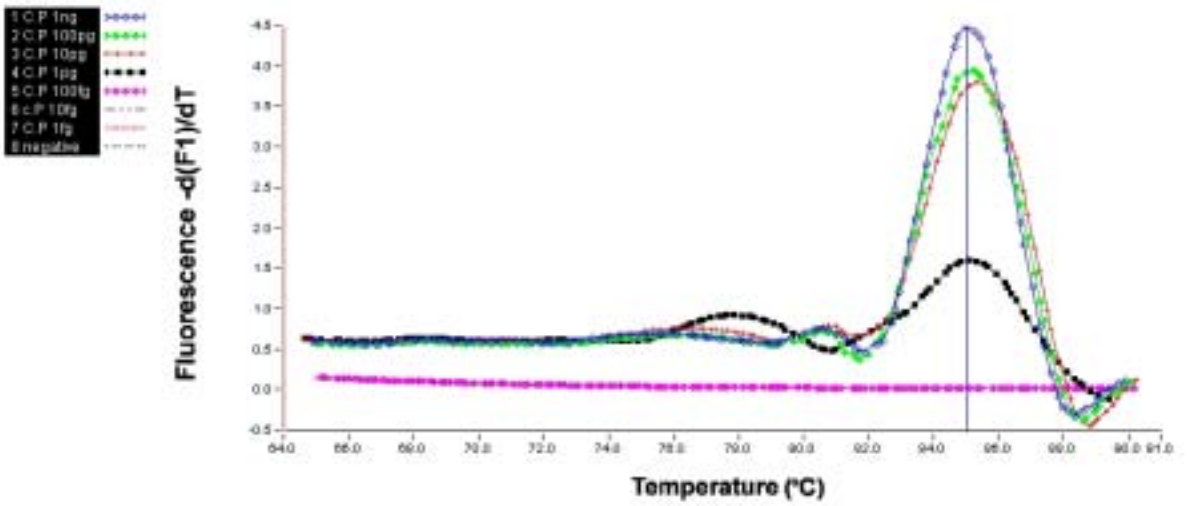
圖十三、使用CGU1及CGU2引子對以real-time PCR分析*C. guilliermondii* (ATCC20862) DNA的專一性。



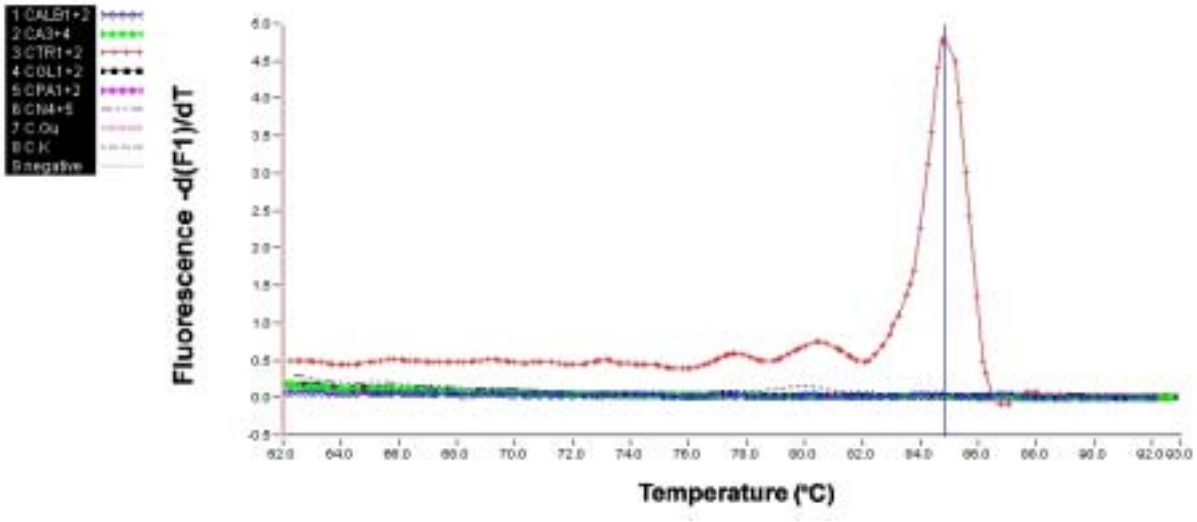
圖十四、使用CGU1及CGU2引子對以real-time PCR分析*C. guilliermondii* (ATCC20862) DNA的敏感度。可偵測*C. guilliermondii* 之DNA濃度為1ng~100fg。



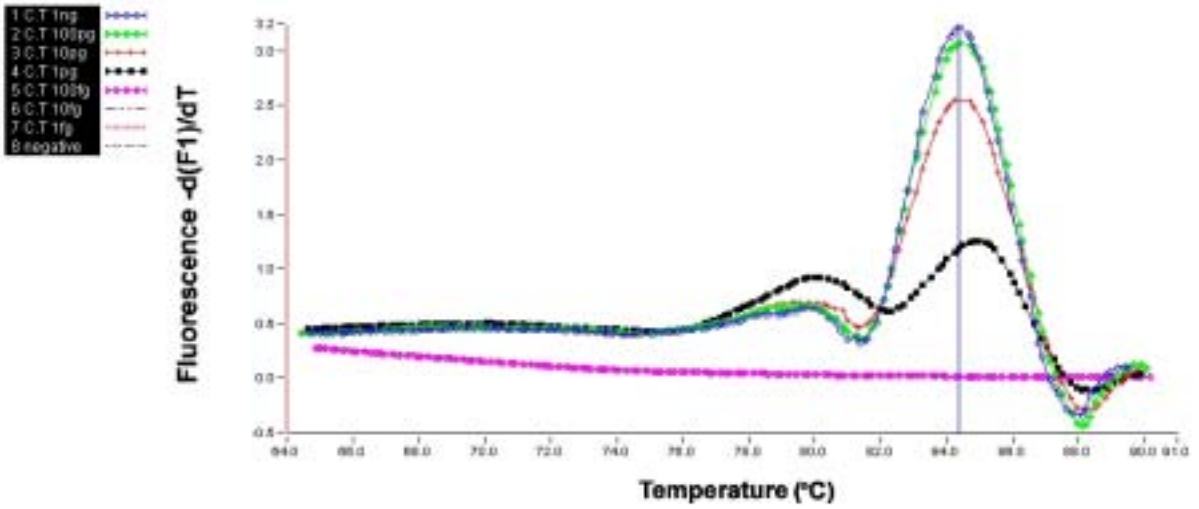
圖十五、使用CPA1及CPA2引子對以real-time PCR分析*C. parapsilosis* (ATCC20515) DNA的專一性。



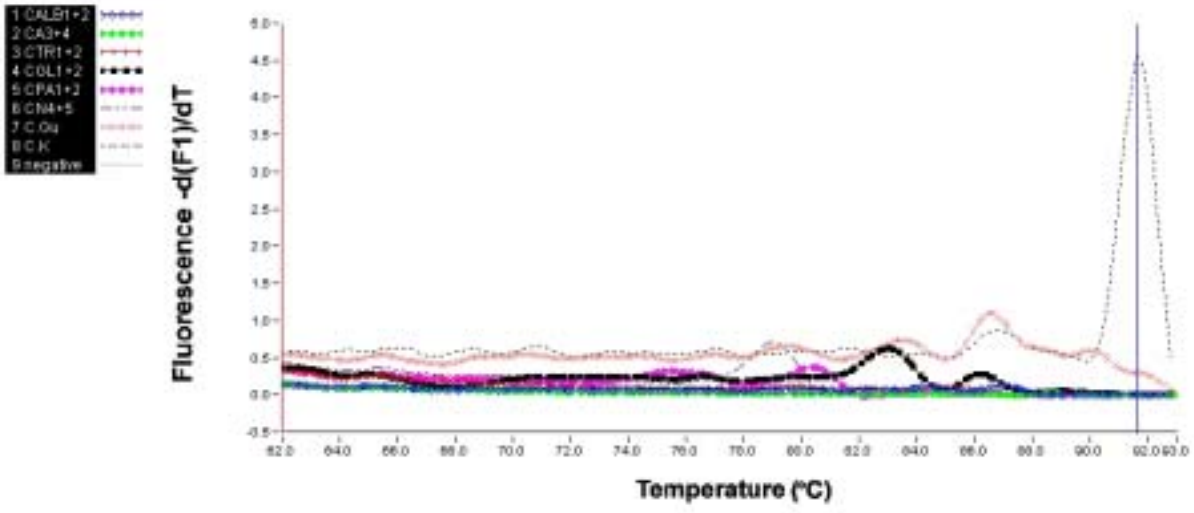
圖十六、使用CPA1及CPA2引子對以real-time PCR分析*C. parapsilosis* (ATCC20515) DNA的敏感度。可偵測*C. parapsilosis*之DNA濃度為1ng~100fg。



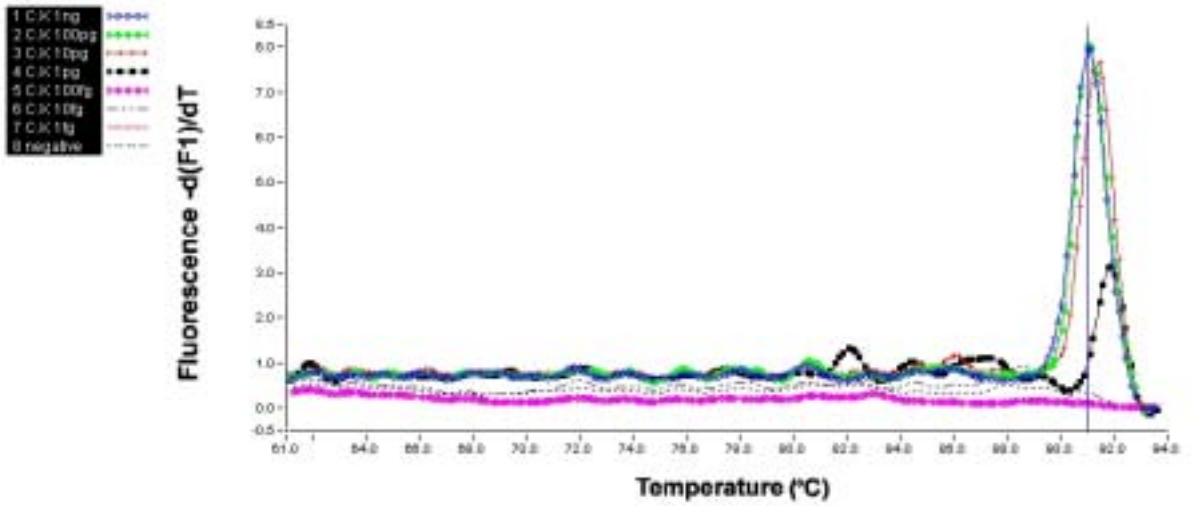
圖十七、使用CTR1及CTR2引子對以real-time PCR分析*C. tropicalis* (ATCC750) DNA的專一性。



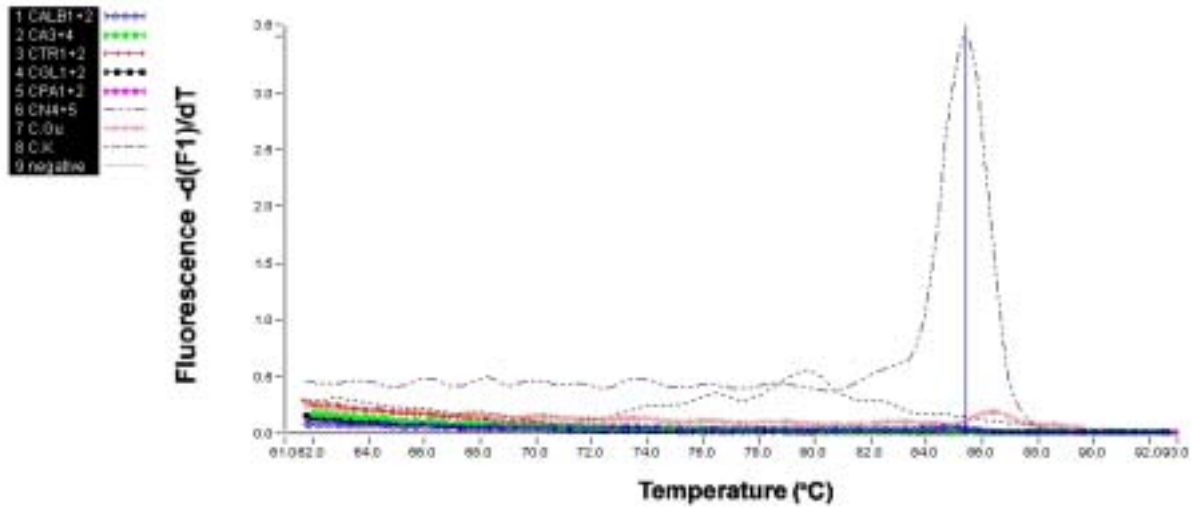
圖十八、使用CTR1及CTR2引子對以real-time PCR分析*C. tropicalis* (ATCC750) DNA的敏感度。可偵測*C. tropicalis*之DNA濃度為1ng~100fg。



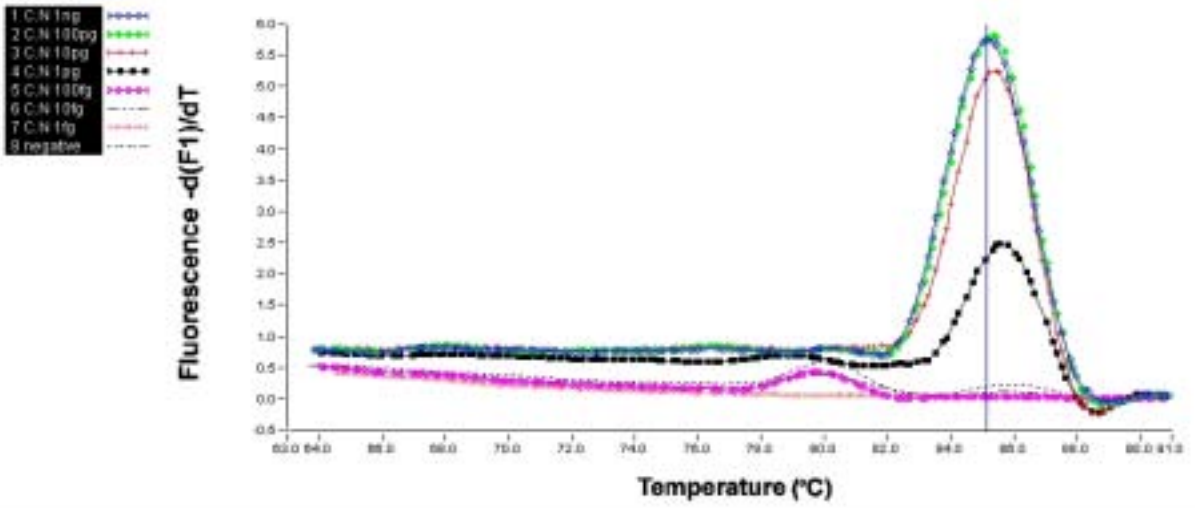
圖十九、使用CKRU1及CKRU2引子對以real-time PCR分析*C. krusei* (ATCC6258) DNA的專一性。



圖二十、使用CKRU1及CKRU2引子對以real-time PCR分析*C. krusei* (ATCC6258) DNA的敏感度。可偵測*C. krusei*之DNA濃度為1ng~100fg。



圖二十一、使用CN4及CN5引子對以real-time PCR分析*Cryptococcus neoformans* DNA的專一性。



圖二十二、使用CN4及CN5引子對以real-time PCR分析*Cryptococcus neoformans* DNA的敏感度。可偵測*Cryptococcus neoformans*之DNA濃度為1ng~100fg。

表一、 PCR-EIA 實驗使用之病原真菌標準及臨床菌株

真菌		病原株數
參考病原株		
Candida albicans	ATCC14053	1
Candida glabrata	ATCC2001	1
Candida parapsilosis	ATCC22019	1
Candida kruse	ATCC6258	1
Candida tropicalis	ATCC750	1
Candida guilliermondii	BCRC21559	1
Cryptococcus neoformas	BCRC22873	1
臨床病原株		
Candida albicans		15
Candida glabrata		8
Candida parapsilosis		6
Candida kruse		4
Candida tropicalis		9
Candida guilliermondii		7
Cryptococcus neoformas		2

表二、Real-time PCR 使用之病原真菌標準及臨床菌株

編號	菌種	地理位置	分離部位
CDC F062	*Candida albicans*	Northern	Urine
CDC F063	*Candida albicans*	Northern	Blood
CDC F064	*Candida albicans*	Northern	Urine
CDC F065	*Candida albicans*	Northern	Blood
CDC F066	*Candida albicans*	Northern	Blood
CDC F087	*Candida albicans*	Western	Urine
CDC F088	*Candida albicans*	Western	Sputum
CDC F089	*Candida albicans*	Western	Sputum
CDC F090	*Candida albicans*	Western	Sputum
CDC F091	*Candida albicans*	Western	Sputum
CDC F092	*Candida albicans*	Western	Wound
CDC F094	*Candida albicans*	Western	Sputum
CDC F095	*Candida albicans*	Western	Sputum
CDC F099	*Candida albicans*	Western	Sputum
CDC F102	*Candida albicans*	Western	Sputum
CDC-F070	*Candida glabrata*	Northern	Urine
CDC-F073	*Candida glabrata*	Northern	Urine
CDC-F075	*Candida glabrata*	Northern	Sputum
CDC-F078	*Candida glabrata*	Northern	Blood
CDC-F093	*Candida glabrata*	Western	Urine
CDC-F096	*Candida glabrata*	Western	Sputum
CDC-F097	*Candida glabrata*	Western	Urine
CDC-F098	*Candida glabrata*	Western	Urine
CDC-F071	*Candida guilliermondii*	Northern	Sputum
CDC-F119	*Candida guilliermondii*	Southern	Skin
CDC-F128	*Candida guilliermondii*	Southern	Sputum
CDC-F171	*Candida guilliermondii*	Western	Tine
CDC-F261	*Candida guilliermondii*	Northern	Blood
CDC-F276	*Candida guilliermondii*	Northern	Nail
CDC-F317	*Candida guilliermondii*	Northern	Blood

CDC-F069	*Candida krusei*	Northern	Urine
CDC-F072	*Candida krusei*	Northern	Urine
CDC-F191	*Candida krusei*	Western	Blood
CDC-F214	*Candida krusei*	Southern	Blood
CDC-F360	*Cryptococcus neoformans*	Eastern	CSF[b]
CDC-F074	*Candida parapsilosis*	Northern	Sputum
CDC-F080	*Candida parapsilosis*	Northern	Wound
CDC-F084	*Candida parapsilosis*	Northern	Blood
CDC-F107	*Candida parapsilosis*	Western	Blood
CDC-F156	*Candida parapsilosis*	Western	Wound
CDC-F159	*Candida parapsilosis*	Western	Urine
CDC-F067	*Candida tropicalis*	Northern	Sputum
CDC-F068	*Candida tropicalis*	Northern	Sputum
CDC-F076	*Candida tropicalis*	Northern	Sputum
CDC-F079	*Candida tropicalis*	Northern	Wound tissue
CDC-F081	*Candida tropicalis*	Northern	Urine
CDC-F082	*Candida tropicalis*	Northern	Urine
CDC-F083	*Candida tropicalis*	Northern	Urine
CDC-F085	*Candida tropicalis*	Northern	Sputum
CDC-F086	*Candida tropicalis*	Northern	Urine
	Candida albicans	ATCC14053 [c]	
	Candida glabrata	ATCC2001	
	Candida guilliermondii	ATCC20862	
Reference	*Candida krusei*	ATCC6258	
isolates	*Candida parapsilosis*	ATCC20515	
	Candida parapsilosis	ATCC22019	
	Candida tropicalis	ATCC750	
	Cryptococcus neoformans		

[a] The geographic location of Taiwan

[b]CSF: cerebral spinal fluid

[c] ATCC, American Type Culture Collection, Manassas, Va.

表三、PCR-AGE 所用寡核甘酸引子序列

真菌	寡核甘酸引子序列（5′ to 3′）	片段大小（鹼基對）
C. albicans	ITS3 GCA TCG ATG AAG AAC GCA GC	~260
	CAL GGA CGT TAC CGC CGC AAG CAA T	
C. glabrata	ITS3 GCA TCG ATG AAG AAC GCA GC	~300
	CGL AAC ACC GAG TTG GTA AAA CCT A	
C. parapsilosis	ITS3 GCA TCG ATG AAG AAC GCA GC	~238
	CPA TGG AAG AAG TTT TGG AGT TTG T	
C. krusei	ITS3 GCA TCG ATG AAG AAC GCA GC	~238
	CKR AAA AGT CTA GTT CGC TCG GGC C	
C. tropicalis	ITS3 GCA TCG ATG AAG AAC GCA GC	~250
	CTR GGC CAC TAG CAA AAT AAG CGT T	
C. guilliermondii	ITS3 GCA TCG ATG AAG AAC GCA GC	~317
	CGU GTT TGG TTG TTG TAA GGC CGG G	
C. neoformans	ITS3 GCA TCG ATG AAG AAC GCA GC	~288
	CN CCG AAG ACT ACC CCA TAG G	

表四、PCR-EIA 所用寡核甘酸引子及探針序列

引子/探針	寡核甘酸引子及探針序列（5′ to 3′）	寡核甘酸標誌
引子		
ITS1	TCC GTA GGT GAA CCT GCG G	28S rDNA universal forward primer
ITS4	TCC TCC GCT TAT TGA TAT GC	28S rDNA universal reverse prime
探針		
ITS3- B	GCA TCG ATG AAG AAC GCA GC	5′-Biotin-labeled universal capture probe
CA	ATT GCT TGC GGC GGT AAC GTC C	5′-end-labeled digoxigenin probe; ITS2 region of *C. albicans*
CG	TAG GTT TTA CCA ACT CGG TGT T	5′-end-labeled digoxigenin probe; ITS2 region of *C. glabrata*
CP	ACA AAC TCC AAA ACT TCT TCC A	5′-end-labeled digoxigenin probe; ITS2 region of *C. parapsilosis*
CK	TTG TTG TCT CGC AAC ACT CGC T	5′-end-labeled digoxigenin probe; ITS2 region of *C. krusei*
CT	AAC GCT TAT TTT GCT AGT GGC C	5′-end-labeled digoxigenin probe; ITS2 region of *C. tropicalis*
GU	CCC GGC CTT ACA ACA ACC AAA C	5′-end-labeled digoxigenin probe; ITS2 region of *C. guilliermondii*
CN	CCT ATG GGG TAG TCT TCG G	5′-end-labeled digoxigenin probe; ITS2 region of *C. neoformans*

表五、 Real-time PCR 所使用之引子對序列

菌種	引子	序列 (5’ →3’)	增幅子大小 (bp)	文獻或資料庫號碼
All fungi	ITS1	TCCGTAGGTGAACCTGCGG	Variable[b]	M27607
	ITS2	GCTGCGTTCTTCATCGATGC		AX592668
	ITS3	GCATCGATGAAGAACGCAGC		AX592669
	ITS4	TCCTCCGCTTATTGATATGC		D89886
C. albicans	CALB1	TTTATCAACTTGTCACACCAGA	~273	L47111
	CALB2	ATCCCGCCTTACCACTACCG		L28817
C. glabrata	CGL1	TTATCACACGACTCGACACT	~423	AB032177
	CGL2	CCCACATACTGATATGGCCTACAA		AF167993
C. guilliermondii	CGU1	GCATCGATGAAGAACGCAGC	~315	AX592669, (22)
	CGU2	GTTTGGTTGTTGTAAGGCCGGG		
C. krusei	CKRU1	GCATCGATGAAGAACGCAGC	~258	AX592669, (21)
	CKRU2	AAAAGTCTAGTTCGCTCGGGCC		
C. parapsilosis	CPA1	GCCAGAGATTAAACTCAACCAA	~300	AF287909
	CPA2	CCTATCCATTAGTTTATACTCCGC		L47109
C. tropicalis	CTR1	CAATCCTACCGCCAGAGGTTAT	~357	AF287910,
	CTR2	TGGCCACTAGCAAAATAAGCGT		AF268095
C. neoformans	CN5	GAAGGGCATGCCTGTTTGAGAG	~136	M94516
	CN4	ATCACCTTCCCACTAACACATT		M94517

[a] Odd-numbered primers are forward primers, and even-numbered primers are reverse primers.

[b] The ITS1-2, ITS3-4 amplicon size vary with each organism.

表六、以免疫酵素測試法分析種別專一性核酸探針偵測病原真菌之特異性。

種別	A_{650} 平均值						
	CA	CG	CP	CK	CT	GU	CN
C. albicans	**2.487**	0.083	0.072	0.055	0.094	0.076	0.063
C. glabrata	0.152	**1.845**	0.075	0.049	0.073	0.078	0.062
C. parapsilosis	0.083	0.070	**2.685**	0.053	0.077	0.120	0.061
C. krusei	0.086	0.075	0.076	**0.874**	0.081	0.092	0.066
C. tropicalis	0.087	0.070	0.074	0.047	**2.904**	0.076	0.081
C. guilliermondii	0.079	0.054	0.053	0.057	0.055	**1.507**	0.047
C. neoformans	0.071	0.056	0.056	0.048	0.055	0.058	**1.216**

臨界值（cutoff Value）為陰性對照平均光密度值(OD)的 0.2 倍＝0.267

表七、用 *C. albicans* 全細胞評估 PCR-EIA 的敏感性

每 50　l 樣本所含細胞數	**用 *C. albicans* 專一性探針 CA 的 A_{650} 值**
1000	**1.301**
100	**0.919**
10	**0.273**
1	**0.072**
＜1	**0.070**

臨界值（cutoff Value）為陰性對照平均光密度值(OD)的0.2倍＝0.260

表八、用 *C. albicans* 萃取 DNA 評估 PCR-EIA 的敏感性

每 50 μl 樣本所含 DNA 量	用 ***C. albicans*** **專一性探針 CA 的 A_{650} 值**
1 ng	**1.0315**
100 pg	**0.954**
10 pg	**0.6775**
1 pg	**0.313**
100 fg	**0.1**
10 fg	**0.1215**
1 fg	**0.082**

臨界值（cutoff Value）為陰性對照平均光密度值(OD)的0.2倍＝0.248

表九、 以泛真菌引子對 PCR 增幅出產物之平均最高 T_m 值

Fungal SpeciesTested	(no.of isolates)	Average peak T_m ± SD with ITS1-4 primers	
		ITS1	ITS2
Candida albicans	(n = 16)	83.90 ± 0.35	85.98 ± 0.14
Candida glabrata	(n = 9)	87.97 ± 0.1	84.72 ± 0.19
Candida guilliermondii	(n = 8)	83.13 ± 0.18	84.27 ± 0.15
Candida krusei	(n = 5)	81.03 ± 0.24	90.18 ± 0.07
Candida parapsilosis	(n = 8)	79.88 ± 0.25	84.68 ± 0.20
Candida tropicalis	(n = 10)	80.81 ± 0.17	83.99 ± 0.17
Cryptococcus neoformans	(n = 2)	81.74 ± 0.25	86.19 ± 0.11

表十、 以特異性引子 PCR 增幅出產物之平均最高 Tm 值

Fungal species Tested	(No.)	$Tm \pm$ SD
C. albicans	(n = 16)	85.71 ± 0.13
C. glabrata	(n = 9)	84.02 ± 0.14
C.guilliermondii	(n = 8)	84.01 ± 0.28
C. krusei	(n = 5)	90.32 ± 0.09
C. parapsilosis	(n = 8)	84.43 ± 0.16
C. tropicalis	(n = 10)	84.03 ± 0.13
C. neoformans	(n = 2)	84.47 ± 0.29

誌謝

本計畫擬向提供臨床酵母菌株及其相關資料之國家衛生研究院羅秀容博士(TsaryI 計畫)及 22 家醫院致最大謝意，這些醫院為： 林口長庚醫院, 基隆長庚醫院, 新竹醫院, 羅東博愛醫院, 聖瑪麗醫院, 台北市立陽明醫院, 台北市立仁愛醫院, 桃園綜合醫院, 台安醫院,辜基金會孫逸仙癌症中心, 三軍總醫院, 光田綜合醫院, 台中榮民總醫院, 仁愛綜合醫院, 奇美醫院, 高雄醫學院附設醫院, 高雄三軍醫院, 台南市立醫院, 高雄榮民總醫院, 花蓮佛教慈濟綜合醫院, 馬偕醫院台東分院.

附錄：本計畫產出著作發表成果

i. 海報論文

Min-Chih Hsu, Guo-Wei Chen, Mei-Hui Liao, Yu-Hui Lin, Chi-Hwa Yang, Shu-Ying Li (2003)Application of Real-time PCR Method for the Identification of Pathogenic Fungi. Current Status and Challenge of Infectious Diseases in Asia. Feb. 21-22. “Current Status & Challenge of Infectious Diseases in Asia”研討會(2003 年二月、台南成大)

ii. 海報論文

Mei-Hui Liao, Yu-Hui Lin, Min-Chih Hsu, Guo-Wei Chen, Chi-Hwa Yang, Shu-Ying Li (2003) Rapid Molecular Identification of Fungal Pathogens. Current Status and Challenge of Infectious Diseases in Asia. Feb. 21-22.
“Current Status & Challenge of Infectious Diseases in Asia” 研討會(2003 年二月、台南成大)

iii. SCI 論文

Species identification of medically important fungi by use of real-time LightCycler PCR. Min-Chih Hsu, Kuo-Wei Chen, Hsiu-Jung Lo, Yee-Chun Chen, Mei-Hui Liao, Yu-Hui Lin, and Shu-Ying Li J Med Microbiol 2003. 52: 1071-1076.

iv. 疫情報導

李淑英, 廖美惠, 林育徽, 徐敏智, 陳國緯(2003)利用聚合鏈連鎖反應—酵素免疫分析法快速鑑定病原真.疫情報導 19(7):366-380。

Journal of Medical Microbiology (2003), 52, 1071–1076 DOI 10.1099/jmm.0.05302-0

Species identification of medically important fungi by use of real-time LightCycler PCR

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Invasive fungal infection has become a major cause of morbidity and mortality in immunocompromised patients. Rapid identification of pathogenic fungi to species level is critical for disease treatment. A real-time LightCycler assay aiming at rapid detection and species identification of pathogenic fungi from clinical isolates was developed. Template DNAs of different species were amplified and detected in real time by employing SYBR Green fluorescent dye. The target sequences for species-level detection were located between the 18S and 28S rDNA. Seven fungal species encountered frequently in the clinical setting, *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis*, *Candida tropicalis*, *Candida guilliermondii* and *Cryptococcus neoformans*, could be discriminated by species-specific primers and confirmed by melting-curve analyses. The range of linearity was from 1 ng to 1 pg (μl^{-1} water) and the sensitivity was 1 pg fungal DNA μl^{-1}. Identification by this real-time PCR method matched biochemical identification for all 58 clinical strains. Therefore, the method is simple, rapid and sensitive enough for detection and identification of several fungal species.

INTRODUCTION

Invasive fungal infections have become major causes of morbidity and mortality among immunocompromised patients (Dasbach *et al.*, 2000; Ellis *et al.*, 2001), such as some neutropenic patients with haematological malignancies and recipients of allogenic bone marrow transplants (Denning, 1998) as well as individuals with AIDS (Mitchell & Perfect, 1995). *Candida* species are now the fourth most frequent cause of nosocomial blood-stream infections in critically ill patients in the United States. At a teaching hospital in Taiwan, yeast infection was identified as the leading cause of nosocomial infection (Chen *et al.*, 1997). The increasing prevalence of yeast infections highlights the need for simple and rapid methods to identify clinically important fungi in a microbiological laboratory.

Each *Candida* species has a different degree of susceptibility to common antifungal agents. For instance, *Candida krusei* is innately resistant and *Candida glabrata*, *Candida guilliermondii* and *Candida dubliniensis* are less susceptible to fluconazole than other *Candida* species (Orozco *et al.*, 1998; Piemonte *et al.*, 1996). Emergence of secondary resistance in *Candida lusitaniae* to amphotericin B has also been observed and monitored closely (Pfaller *et al.*, 2003). Current recommendations suggest that invasive fungal infections, such as candidiasis and aspergillosis, should be treated empirically, because the current diagnoses are difficult and time-consuming (Rex *et al.*, 2000; Stevens *et al.*, 2000). However, there is great concern that such practice would result in the emergence of resistant fungal pathogens.

As more and more alternative antifungal agents with various spectra of activities are developed and become available, treatment according to accurate diagnosis has become even more important. Therefore, rapid species identification will be more critical for effective disease therapy and control (Polak, 2003). Conventional diagnostic tests, such as blood culture and biochemical tests, which lack sufficient sensitivity and specificity for early diagnosis of invasive fungal infections, may often require 2 or more days and may be inaccurate (Espinel-Ingroff *et al.*, 1998; Goodwin *et al.*, 1992; Hazen, 1995). Serological tests have certain limitations, e.g. antibody response may be lacking or varied, since the patients most at risk of fungal infections are often immunosuppressed. As for antigen tests, there are no widely accepted *Candida* antigen tests and most cannot differentiate among *Candida* species. Other tests, such as those for cryptococcal, aspergillus and histoplasmal antigen, are often hampered by low serum antigen concentrations (Morrison & Lindsley, 2002; Yeo & Wong, 2002).

Therefore, diagnostic assays based on *in vitro* amplification and detection of fungal DNA have been developed, among which PCR methods are particularly promising because of their high specificity and sensitivity. A number of studies have described restriction fragment length polymorphism, PCR amplification and hybridization with species-specific probes, amplicon size differences (Chen *et al.*, 2001; Fujita *et al.*, 2001; Henry *et al.*, 2000) or other methods to identify unique DNA sequences (Hopfer *et al.*, 1993; Kappe *et al.*, 1998; Martin *et al.*, 2000; Turenne *et al.*, 1999). Although these published PCR methods are quite useful for identification of fungal species, they still require a minimum of several hours for DNA amplification and visualization. More recently, real-time PCR techniques have been developed for the detection of fungal pathogens such as *Candida* species, *Cryptococcus neoformans* and *Aspergillus* species. All these assays demonstrate sensitivities better or at least comparable to previously described PCR methods. Real-time PCR assays dramatically decrease the risk of false-positive results, because the PCR and detection systems are coupled and conducted in a closed system and no laborious post-PCR analyses are required. Various real-time PCR platforms have been developed. The signal to be analysed can be generated by double-stranded DNA-specific dyes, such as SYBR Green, or by sequence-specific fluorescence energy transfer probes. A couple of exonuclease-based TaqMan PCR assays, capable of rapid identification and speciation of six *Candida* species (Guiver *et al.*, 2001) or *Aspergillus fumigatus* (Costa *et al.*, 2002; Kami *et al.*, 2001), have been described. DNA detection methods for *Candida albicans*, *A. fumigatus* and *Cryptococcus neoformans* using the LightCycler have also been reported (Bialek *et al.*, 2002; Loeffler *et al.*, 2000; Spiess *et al.*, 2003). White *et al.* (2003) described real-time and high-sensitivity detection of seven *Candida* species using the LightCycler system; however, they were not able to speciate them.

Table 1. Clinical and reference strains used in this study

Strain	Source of isolation	Strain	Source of isolation
Candida albicans		*Candida guilliermondii*	
ATCC 14053	Reference strain	CDC-F276	Nail
CDC-F062	Urine	CDC-F317	Blood
CDC-F063	Blood	*Candida krusei*	
CDC-F064	Urine	ATCC 6258	Reference strain
CDC-F065	Blood	CDC-F069	Urine
CDC-F066	Blood	CDC-F072	Urine
CDC-F087	Urine	CDC-F191	Blood
CDC-F088	Sputum	CDC-F214	Blood
CDC-F089	Sputum	*Cryptococcus neoformans*	
CDC-F090	Sputum	Duke215	Reference strain
CDC-F091	Sputum	CDC-F360	Cerebrospinal fluid
CDC-F092	Wound	*Candida parapsilosis*	
CDC-F094	Sputum	ATCC 20515	Reference strain
CDC-F095	Sputum	ATCC 22019	Reference strain
CDC-F099	Sputum	CDC-F074	Sputum
CDC-F102	Sputum	CDC-F080	Wound
Candida glabrata		CDC-F084	Blood
ATCC 2001	Reference strain	CDC-F107	Blood
CDC-F070	Urine	CDC-F156	Wound
CDC-F073	Urine	CDC-F159	Urine
CDC-F075	Sputum	*Candida tropicalis*	
CDC-F078	Blood	ATCC 750	Reference strain
CDC-F093	Urine	CDC-F067	Sputum
CDC-F096	Sputum	CDC-F068	Sputum
CDC-F097	Urine	CDC-F076	Sputum
CDC-F098	Urine	CDC-F079	Wound tissue
Candida guilliermondii		CDC-F081	Urine
ATCC 20862	Reference strain	CDC-F082	Urine
CDC-F071	Sputum	CDC-F083	Urine
CDC-F119	Skin	CDC-F085	Sputum
CDC-F128	Sputum	CDC-F086	Urine
CDC-F171	Tinea	CDC-F085	Sputum
CDC-F261	Blood	CDC-F086	Urine

In this paper, we describe a simple real-time PCR assay with the LightCycler system employing species-specific primers and SYBR Green fluorescent dye for detection and species identification of fungal strains. This assay offers the advantage that the conventional PCR can be easily adapted to real-time format without the need for complicated probe design. The assay has been shown to be amply specific and sensitive and to facilitate the detection procedure significantly.

METHODS

Fungal strains. Eight reference strains and 50 clinical strains of *Candida albicans* ($n = 16$), *Candida glabrata* ($n = 9$), *Candida guilliermondii* ($n = 8$), *Candida krusei* ($n = 5$), *Candida parapsilosis* ($n = 8$), *Candida tropicalis* ($n = 10$) and *Cryptococcus neoformans* ($n = 2$) were used in this study (Table 1). Taiwan Surveillance of Antimicrobial Resistance of Yeasts (Lo *et al.*, 2001) collected clinical strains isolated from different body sites and from 22 hospitals located in different geographical locations in Taiwan from 15 April to 15 June 1999. Identification of fungal strains was done by germ-tube assay followed by VITEK yeast biochemical card and API-32C systems. Fungi were cultured on Sabouraud dextrose agar (SDA) plates for 72 h at 37 °C.

DNA extraction. DNA was extracted by using the PUREGENE DNA purification kit (Gentra). Briefly, two loops of fungal biomass from a 2- to 3-day-old culture on SDA were transferred to a 5-ml sterile tube with parafilm seals and re-suspended in 2 ml PBS containing 10–15 µl lyticase and the mixture was incubated overnight at 37 °C. The samples were centrifuged at 13 000 *g* for 3 min and the supernatant was removed. Cell lysis solution (2 ml) was added to the cell pellet and gently pipetted up and down to lyse the cells. Next, 1 ml protein precipitation solution was added to the cell lysate and vortexed vigorously at high speed for 20 s and then centrifuged at 13 000 *g* for 10 min. The supernatant was mixed with 100 % isopropanol to precipitate DNA. The DNA was washed with 70 % ethanol, air-dried and dissolved in 50 µl DNA hydration solution. DNA concentrations were measured with a spectrophotometer (A_{260}) and DNA samples were stored at −80 °C until used.

LightCycler-based PCR. The LightCycler PCR and detection system (Roche Diagnostics) was used for amplification and quantification. PCR was performed in glass capillaries and cycling was achieved by alternating heated air and air of ambient temperature, which ensures rapid equilibration between the air and the reaction components due to the high surface-to-volume ratio of the capillaries. The locations and sequences of the species-specific primers (CALB, CGL, CPA, CTR, CGU, CKRU and CN) are shown in Fig. 1 and Table 2 (Lindsley *et al.*, 2001; Luo & Mitchell, 2002). For amplicon detection, the LightCycler FastStart DNA Master SYBR Green kit was used as described by the manufacturer. The PCR mixture (20 µl) contained Taq polymerase, 1× LightCycler reaction buffer, 3 mM magnesium chloride and 0·5 µM primers. Template DNA was added at a final concentration of 1 ng per 20 µl reaction mixture. Samples were run in parallel by performing 35 cycles of repeated denaturation (5 s at 95 °C), annealing (5 s at 58 °C) and chain extension (25 s at 72 °C). This step was followed by a melting-curve analysis from 60 to 95 °C and, afterwards, cooling to 40 °C. The PCR was completed within 45 min. The PCR process was then monitored by fluorescence quantification of the DNA-binding dye SYBR Green I dye for the detection of double-stranded DNA.

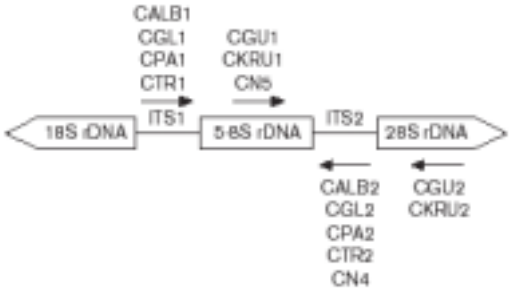

Fig. 1. Specific primer sites for the real-time PCR.

Agarose gel electrophoresis. Gel electrophoresis was conducted in TBE buffer (0·1 M Tris/HCl, 0·09 M boric acid, 1 mM EDTA, pH 8·4) at 100 V cm^{-1} for 50 min in gels composed of 2·0 % (w/v) agarose (BioWhittaker) and stained in 0·5 µg ethidium bromide ml^{-1} for 15 min followed by washing for 15 min with distilled water.

RESULTS AND DISCUSSION

The increasing frequency of invasive fungal infections and the high mortality associated with disseminated fungal diseases have underscored the importance of rapid detection of pathogenic fungi. Prompt detection and accurate speciation may help to improve fungal disease management as a whole and lead to more rational use of antifungals. Traditional identification methods based on phenotypic features are often time-consuming and depend largely on the skill and experience of the technician. Therefore, we evaluated the feasibility of LightCycler PCR amplification of the rDNA region followed by melting-curve analysis for identification of clinically important yeasts.

The specificity of LightCycler amplification was tested by annealing the *Candida albicans*-specific primers CALB1 and CALB2 with DNA extracted from *Candida albicans* ATCC 14053. The detection limit was about 1 pg μl^{-1} (Fig. 2). Similar results were observed with DNA of other species, i.e. the specific primers for each fungal species react only with DNA from the homologous fungal species (data not shown).

The range of linearity was tested by amplification of 10-fold serial dilutions of purified *Candida albicans* ATCC 14053 DNA with fluorescence plotted against number of cycles. Linearity was achieved over 4 logs of input fungal DNA amount, from 1 ng to 1 pg μl^{-1} (Fig. 3).

Compared with bacteraemia or viraemia, fungaemia has much lower pathogen load. Therefore, high-sensitivity detection is pivotal for timely management of fungal diseases. The sensitivity of the assays was evaluated by amplifying DNAs of six *Candida* species and *Cryptococcus neoformans* by their respective primers with the LightCycler, and the detection limit of this method was about 1 pg DNA μl^{-1}. Serially diluted samples of reference strains ranging from 1 ng to 1 pg DNA μl^{-1} showed a single band with the specific primers in all cases (Fig. 4).

In order to determine the utility of the LightCycler species-specific amplification method for accurate identification of fungal species, analyses of clinical strains were conducted.

Table 2. Primers for PCR amplification

Odd-numbered primers are forward primers and even-numbered primers are reverse primers.

Primer	Sequence (5′–3′)	Amplicon (bp)	Reference/GenBank accession no.
Candida albicans			
CALB1	TTTATCAACTTGTCACACCAGA	~273	L47111
CALB2	ATCCCGCCTTACCACTACCG		L28817
Candida glabrata			
CGL1	TTATCACACGACTCGACACT	~423	AB052177
CGL2	CCCACATACTGATATGGCCTACAA		AF167993
Candida guilliermondii			
CGU1	GCATCGATGAAGAACGCAGC	~315	AX592669
CGU2	GTTTGGTTGTTGTAAGGCCGGG		Elie *et al.* (1998)
Candida krusei			
CKRU1	GCATCGATGAAGAACGCAGC	~258	AX592669
CKRU2	AAAAGTCTAGTTCGCTCGGGCC		Elie *et al.* (1998)
Candida parapsilosis			
CPA1	GCCAGAGATTAAACTCAACCAA	~300	AF287909
CPA2	CCTATCCATTAGTTTATACTCCGC		L47109
Candida tropicalis			
CTR1	CAATCCTACCGCCAGAGGTTAT	~357	AF287910
CTR2	TGGCCACTAGCAAAATAAGCGT		AF268095
Cryptococcus neoformans			
CN5	GAAGGGCATGCCTGTTTGAGAG	~136	M94516
CN4	ATCACCTTCCCACTAACACATT		M94517

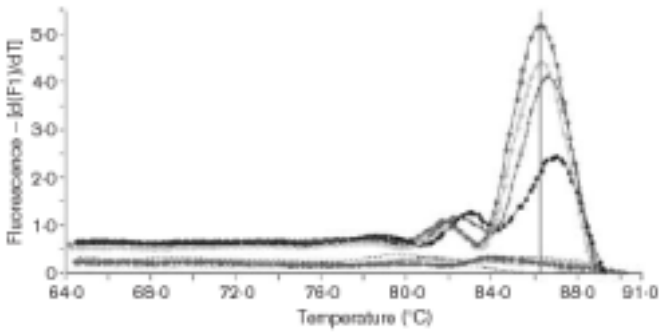

Fig. 2. Sensitivity of the *Candida albicans* ATCC 14053 DNA-specific primers CALB1 and CALB2. Characteristic dose-dependent melting peaks were observed in the presence of DNA from *Candida albicans* ATCC 14053 [1 ng (◇) to 1 pg (■) μl^{-1}].

The identification results from our real-time PCR method matched completely with biochemical identification results for all 58 tested samples. Characteristic peak T_m for species-specific primers with their respective fungal species were obtained by melting-curve analyses (Table 3).

Targets for species-level detection of fungal pathogens include the 18S rDNA, mitochondrial DNA (Yamada *et al.*, 2002), the internal transcribed spacer (ITS) regions and many other genes (Kanbe *et al.*, 2002). rDNA offers distinct advantages over other molecular targets because of greatly increased sensitivity due to the existence of approximately 100 copies per genome. Many *Candida* species can be differentiated by analysis of fragment length variation in ITS1 (Chen *et al.*, 2001) or ITS2 (De Baere *et al.*, 2002). The LightCycler system offers another advantage of analysis of the melting temperature of amplicons. The melting temperature of the amplicon is dependent on the G+C content, sequence length and compositional variation in the nucleotide bases. Each fungal species has a characteristic T_m, which helps further to confirm its identity.

In conclusion, the real-time LightCycler PCR assay combines rapid amplification of DNA with real-time species determination. The routine block cycler PCR protocol can be easily transferred and adapted to the real-time protocol. This method is simple, rapid and sensitive and can therefore streamline the flow of diagnostic laboratory work.

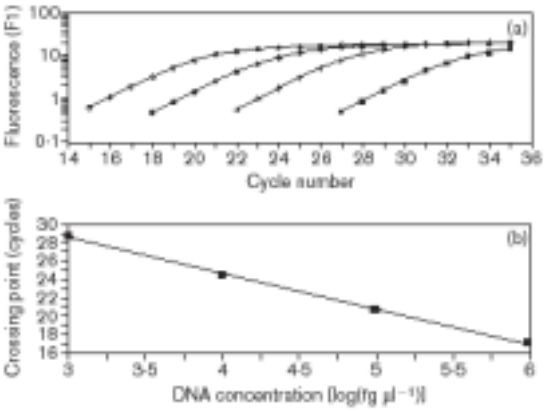

Fig. 3. (a) Quantification of serially diluted *Candida albicans* ATCC 14053 DNA [1 ng to 1 pg µl^{-1}] with CALB1 and CALB2 primers using the LightCycler software. (b) Log DNA concentration (fg µl^{-1}) against fluorescence. Slope (−3·924), *y*-intercept (40·31), mean squared error (0·100) and regression coefficient (−1·00) of the standard curve were calculated.

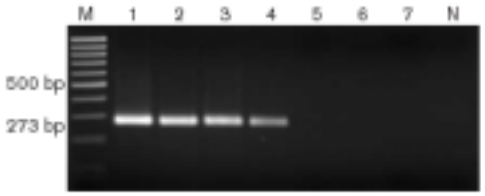

Fig. 4. Agarose electrophoretic gel of LightCycler-amplified DNA from *Candida albicans* ATCC 14053, showing a single, specific band of 275 bp. Lanes: M; marker; 1, 1 ng µl^{-1}; 2, 100 pg µl^{-1}; 3, 10 pg µl^{-1}; 4, 1 pg µl^{-1}; 5, 100 fg µl^{-1}; 6, 10 fg µl^{-1}; 7, 1 fg µl^{-1}; N, negative control (distilled water).

Table 3. T_m values of DNA products amplified with species-specific primers

Species tested	*n*	T_m (mean ± SD) (°C)
Candida albicans	16	85·71 ± 0·13
Candida glabrata	9	84·02 ± 0·14
Candida guilliermondii	8	84·01 ± 0·28
Candida krusei	5	90·32 ± 0·09
Candida parapsilosis	8	84·43 ± 0·16
Candida tropicalis	10	84·03 ± 0·13
Cryptococcus neoformans	2	84·47 ± 0·29

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疫情報導

利用聚合酶連鎖反應– 酵素免疫分析法快速鑑定病原真菌

摘　要

過去二十年以來對病原真菌易罹性個體不斷增加，助長了病原真菌的感染趨勢。正確的種別鑑定對於病原真菌的診療及防治十分重要。傳統的型態及生化鑑定方法費時且易流於主觀，因此，本文評估發展 PCR-EIA 方法應用於鑑定臨床上重要真菌之可行性。原理為將 Candida albicans, Candida glabrata, Candida guilliermondii, Candida krusei, Candida parapsilosis, Candida tropicalis and Cryptococcus neoformans 等真菌全細胞或抽取的 DNA 用泛真菌引子 ITS1 及 ITS4 增幅 rRNA 片段，將增幅片段與 digoxigenin 標誌的種別專一性探針雜合後，再以 EIA 進行呈色反應。本方法之專一性 100%，靈敏度與 PCR-電泳凝膠法相當可達 10 CFU/50 μl 或 1pg/50 μl。以 51 株臨床及 7 株標準菌株測試結果，與綜合 germ tube , Viteck 及 API20C 的生理生化鑑定法結果符合度達 100%。本 PCR-EIA 方法提供了傳統鑑定方法以外的另一種選擇。

前　言

近年來因人類免疫不全病毒(HIV)之感染及癌症病人的增加，抗生素及類固醇之廣泛使用，免疫抑制治療、器官移植及重症照護等醫療行為的長足進步及人口老化等因素、導致免疫功能不全之宿主大量增加，助長了致病性真

菌感染之趨勢[1;2]。除上述機緣性感染之案例外，肇因於都市的發展、人口遷移及自然災害等因素，病原真菌對於健康個體之威脅性亦與日俱增，使得真菌感染之嚴重性漸獲重視[3-5]。

真菌感染的預後差且致死率高。病原真菌對免疫缺損宿主的感染已成爲嚴重的問題。在美國，念珠菌感染(candidiasis)已成爲院內感染的第四位。在台灣，念珠菌引發的院內感染亦高居不下[6;7]。在菌種分析方面，常見的有 Candida albicans 、Candida tropicalis、Candida parapsilosis、Candida glabrata、Candida guilliermondii 及 Candida krusei [6]。其中抗藥性菌株的崛起如對 azole 藥物完全具抗性的 C. krusei 的出現及 C. glabrata、C. tropicalis 抗藥性的增加，更增加問題的棘手性[8;9]。此外，因免疫缺損患者增加、尤加利樹的植被、鴿子的養殖等問題 Cryptococcus neoformans 引起的新型隱球菌亦不容忽視[10;11]。真菌的診斷不易而抗真菌藥物的選擇又少，不同種別對藥物的感受性不同，正確的種別鑑定有助於臨床正確投藥，預防抗藥性問題的出現。

真菌在診斷上頗爲困難，正確而及時的診斷對病害診治具有莫大的幫助[12]，且有助於瞭解真菌性病害的實際嚴重性及經濟上衝擊。真菌已知具有病原性的約 150 種，傳統鑑別診斷方法一般結合培養法，傳統的型態及生化鑑定方法。培養法費時且易流於主觀。生化生理檢測法如用 API20、Vitek、Rapid 檢測法及免疫檢測法來檢測檢體之真菌抗原、抗體及代謝物。這些方法的敏感度及專一性仍有改善的空間，因此需輔助以分子生物方法以達能快速、精確及敏感偵測之目的。在疾病診斷及鑑別上聚合酵素連鎖反應 (polymerase chain reaction, PCR) 最爲常見，原理爲利用特定的寡核甘酸引子增幅一特定之 DNA 片段並以電泳凝膠、EIA[13;14]、長短定序分析[15;16]或螢光及時偵測方法[17]加以檢測。真菌上最常用的方法爲利用真菌粒腺體 DNA (mitochondrial DNA)、核醣體 DNA (ribosomal DNA，rDNA) 5S、18S、28S 基因或間隔轉錄區(ITS1-4)片段序列之特性，從中尋找核甘酸序列差異，設計專一性引子以鑑別不同種之病原真菌。核醣體 DNA 由於複本數多(> 100)故偵測靈敏度較高。

本文評估發展 PCR-EIA 方法應用於鑑定臨床上重要真菌 C. albican, C. glabrata, C. guilliermondii, C. krusei, C. parapsilosis, C. tropicalis and C. neoformans 之可行性。原理爲利用泛真菌引子(pan-fungal primers) ITS1 及 ITS4 增幅真菌 5.8S ribosomal DNA (rDNA) 3' 末端及 28S rDNA 5'末端, 中間包含 internally transcribed spacer 2 (ITS2)序列, 產生 600-到 800-bp 片段。接著以用 digoxigenin 標誌針對 ITS2 的種別探針與 PCR 增幅出之產物做雜合，再以 EIA 呈色原理使有雜合的反應呈色。

材料與方法

1. 病原株：Candida albicans (ATCC14053), Candida glabrata (ATCC2001), Candida krusei (ATCC6258), Candida parapsilosis (ATCC20515), Candida parapsilosis (ATCC22019), Candida tropicalis (ATCC750) 標準菌株由台大醫院陳宜君醫師提供, Candida guilliermondii (BCRC21559) , Cryptococcus neoformans（BCRC22873） 標準菌株由成大醫技系張長泉教授、51 株臨床菌株來自國家衛生研究院從各大醫院收集之臨床菌株 (TSARY I 計畫)[18] (表三)。

2. 菌株之分離及培養：由臨床病患檢體分離培養該菌，菌株用 Sabouraud dextrose agar (SDA)培養於 37℃,72 小時。

3. 菌株之型態及生理生化鑑定：採用常用之 API 20C AUX stripe 或 VITEK automatic systems 及 Germ-tube assay.

4. PCR 引子之設計：參考文獻並針對 ITSII 之差異性比對之結果，設計各真菌之引子及核酸探針[13;14;19]。

＊PCR-電泳凝膠(PCR-AGE)：使用之泛真菌 ITS3 及種別引子(CAL, CGL, CPA, CKR, CTR, CGU, CN) 增幅 ITS2 區域，詳細序列見表二。

＊PCR-EIA： 使用之泛真菌 ITS1 及 ITS4 及針對 ITS2 的種別引子/探針詳細序列見表三。種別引子標誌有 digoxigenin，ITS3 序列在 5’ 端標誌有

biotin。詳細序列見表三。引子及探針所針對之 rRNA 部位詳見圖一。

5. DNA 萃取：

酵母菌 DNA 之萃取：取二個接種環的菌量置於 5-ml 無菌試管以 2ml 內含 10-15ml lyticase 的 PBS 充分懸浮; 置於 37℃中隔夜。接著依廠商說明書建議使用 PUREGENE DNA 純化套組(Gentra, Minneapolis, Minnesota, USA)純化 DNA。抽出 DNA 的以光度比色計用 A260 波長測量濃度。DNA 冰存於 -20℃待用。

6. 敏感度測試：

利用純粹培養之 C. albicans 調成 3.0 MacFarland 單位(約爲 1x106 CFU/ml)，接著直接將菌液以 100℃煮 5 分鐘。將煮過的懸浮液做十倍系列稀釋，當做 PCR-AGE 或 PCR-EIA 的 template DNA。

將抽取的 DNA 從 1ng 到 1fg 依序做 10 倍序列稀釋。

7. PCR 增幅：增幅引子使用 ITS1 及 ITS4。PCR 反應容積爲 50 μl，內含 1 μl 待測 DNA，25 μl 2 X PCR 緩衝液（MBI Fermentas 2X PCR Master Mix），0.5 μM 各種引子，其餘加蒸餾水混勻。增幅初始變性反應 94℃ 5 分鐘溫度，35 次循環的變性反應 94℃ 30 秒→黏和 58℃ 30 秒→72℃ 1 分鐘聚合延長反應，最後爲 72℃ 10 分鐘聚合延長反應。PCR 機器使用 PTC-200(MJ research)。試劑製備反應全程均有依據 Kwok 及 Higuchi 的預防污染建議[20]。

8. 瓊脂膠體電泳分析（agarose gel electrophoresis）：以 1.5% 瓊脂膠體 (Seakem® LE agarose, BioWhittaker Molecular Applications, USA) 於1X的TBE 緩衝液(0.1 M Tris, 0.09 M boric acid, 1 mM EDTA [pH 8.4])進行 DNA 電泳分析。跑電泳功率設定爲 100 V，2 小時。每個待測樣本取 5 μl 並以 100-bp DNA 分子量標準片段同時跑做對照。電泳結束後，於每毫升 0.5 μg ethidium bromide (EtBr) 染劑中染 15 分鐘，再以蒸餾水去染。

9. EIA：將泛真菌引子增幅之片段取 10 μl 先加熱 95℃,5 分鐘，接著置於冰上，加入 200 μl 的 hybridization buffer(4× SSC [1× SSC is 0.15 M NaCl plus 0.015

M sodium citrate], pH 7.0, 0.02 M HEPES, 0.002 M EDTA, and 0.15% Tween 20) 及 10ng 的 ITS3-標誌有 biotin 的泛真菌探針和 10ng 的標誌 digoxigenin 的種別探針。混合後置於 37℃、1 小時。將 100 μl 混合液加入事先黏附有 strepavidin 的 96 孔盤，於室溫下震盪(~350 rpm)培養 1 小時後以 0.01M PBS-Tween 洗六次。接著加入 100 μl 的 1：1000 倍稀釋的 horseradish peroxidase 標定的抗 dioxigenin 抗體(150 U/ml; Roche)，於室溫下震盪培養 1 小時後以 0.01M PBS-Tween 洗六次。最後加入 3,3' ,5,5' -Tetra- methylbenzidine (TMB)-H2O2 基質，反應 10 分鐘後，於光度計 (Quant Universal Microplate Spectrophotometer，BIO-TEK INSTRUMENTS, INC. USA)以 650nm 讀取吸光值。

結　果

PCR-電泳凝膠

以 ITS3 及種別專一性引子(CAL, CGL, CPA, CKR, CTR,CGU,CN)針對各種病原真菌做 PCR，產生增幅子片段約爲 200～300bp (圖二)。ITS3 及 CAL 引子可將 C. albicans 增幅出一約 260-bp 片段產物，與其他六種 Candida spp. 的 DNA 均無反應。 ITS3 及 CAP 引子與 C. parapsilosis 可增幅出約 250-bp 的產物與其他六種 Candida spp.的 DNA 均無反應(圖三), 其他種別引子亦僅針對同種的 DNA 會有 PCR 反應，他種的則沒有反應(圖未列出)。

將 C. albicans 調成 106 至 100 cells /ml 不同菌液濃度以 primer ITS3 和 CAL 進行 PCR 反應並測試其敏感度，結果顯示菌液濃度稀釋至 104 cells /ml（10 CFU 每 50μl 反應容積）仍可以見到約 270bp 之產物（圖四）。

PCR-EIA

以標準菌株做試驗顯示，七種真菌均有反應，且這些探針只與同種病原的 DNA 有雜和反應，與他種 DNA 則無雜和呈色反應（表四）。PCR-EIA 結果

顯現種別特異性探針偵測病原真菌之敏感度爲 10 CFU /50 μl（表五）或 1pg/50 μl（表六）。

PCR-AGE vs. PCR-EIA

以 51 株臨床菌株包含 C. albicans (n=16), C. glabrata (n=8), C. parapsilosis (n=6), C. Krusei (n=4), C. tropicalis (n=9), C. guilliermondii (n=7) and C. neoformans (n=2)分別測試 PCR-AGE 及 PCR-EIA 鑑定結果。以 PCR-AGE 或 PCR-EIA 鑑定，51 株鑑定結果均吻合生化檢驗結果。（表六）。

討 論

真菌性病害近年來的顯著增加及其高死亡率，凸顯了真菌致病原 快速及正確鑑別診斷的重要性。隨著抗真菌藥物發展的進展，提供了不同種別及抗藥性投藥的多種選擇，使得正確種別鑑定更形重要。本文所描述的 PCR-EIA 快速檢定方法，可鑑別 7 種臨床上重要真菌，專一性高達 100%，靈敏度高達 10 CFU/ml，所需時間小於 4 小時。以 51 株臨床及 7 株標準菌株測試結果，與綜合 germ tube , Viteck 及 API20C 的生理生化鑑定法結果符合度達 100%。使用的 DNA 模版可使用萃取的 DNA 也可以直接用全菌體。直接用全菌體更可簡化步驟，降低污染的風險。PCR-EIA 方法專一性高、敏感度高且具有可自動化及大量檢體處理之潛力，此外，二種以上的混合感染也能被偵測出來。要言之，本 PCR-EIA 方法提供了傳統鑑定方法以外的另一種選擇，若能與血清、培養、鏡檢等傳統方法做整合，有助於增加檢驗之準確性及效率。

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表一　本實驗使用的病原真菌株

真菌		病原株數
參考病原株		
Candida albicans	ATCC14053	1
Candida glabrata	ATCC2001	1
Candida parapsilosis	ATCC22019	1
Candida kruse	ATCC6258	1
Candida tropicalis	ATCC750	1
Candida guilliermondii	BCRC21559	1
Cryptococcus neoformas	BCRC22873	1
臨床病原株		
Candida albicans		15
Candida glabrata		8
Candida parapsilosis		6
Candida kruse		4
Candida tropicalis		9
Candida guilliermondii		7
Cryptococcus neoformas		2

表二 PCR-AGE 所用寡核甘酸引子序列

真菌	寡核甘酸引子序列（5′ to 3′）	增幅片段大小（鹼基對）
C. albicans	ITS3 GCA TCG ATG AAG AAC GCA GC	~260
	CAL GGA CGT TAC CGC CGC AAG CAA T	
C. glabrata	ITS3 GCA TCG ATG AAG AAC GCA GC	~300
	CGL AAC ACC GAG TTG GTA AAA CCT A	
C. parapsilosis	ITS3 GCA TCG ATG AAG AAC GCA GC	
	CPA TGG AAG AAG TTT TGG AGT TTG T	~238
C. krusei	ITS3 GCA TCG ATG AAG AAC GCA GC	~238
	CKR AAA AGT CTA GTT CGC TCG GGC C	
C. tropicalis	ITS3 GCA TCG ATG AAG AAC GCA GC	~250
	CTR GGC CAC TAG CAA AAT AAG CGT T	
C. guilliermondii	ITS3 GCA TCG ATG AAG AAC GCA GC	~317
	CGU GTT TGG TTG TTG TAA GGC CGG G	
C. neoformans	ITS3 GCA TCG ATG AAG AAC GCA GC	~288
	CN CCG AAG ACT ACC CCA TAG G	

表三 PCR-EIA 所用寡核甘酸引子及探針序列

引子/探針	寡核甘酸引子及探針序列（5′ to 3′）	寡核甘酸標誌
引子		
ITS1	TCC GTA GGT GAA CCT GCG G	28S rDNA universal forward primer
ITS4	TCC TCC GCT TAT TGA TAT GC	28S rDNA universal reverse prime
探針		
ITS3- B	GCA TCG ATG AAG AAC GCA GC	5′-Biotin-labeled universal capture probe
CA	ATT GCT TGC GGC GGT AAC GTC C	5′-end-labeled digoxigenin probe; ITS2 region of *C. albicans*
CG	TAG GTT TTA CCA ACT CGG TGT T	5′-end-labeled digoxigenin probe; ITS2 region of *C. glabrata*
CP	ACA AAC TCC AAA ACT TCT TCC A	5′-end-labeled digoxigenin probe; ITS2 region of *C. parapsilosis*
CK	TTG TTG TCT CGC AAC ACT CGC T	5′-end-labeled digoxigenin probe; ITS2 region of *C. krusei*
CT	AAC GCT TAT TTT GCT AGT GGC C	5′-end-labeled digoxigenin probe; ITS2 region of *C. tropicalis*
GU	CCC GGC CTT ACA ACA ACC AAA C	5′-end-labeled digoxigenin probe; ITS2 region of *C. guilliermondii*
CN	CCT ATG GGG TAG TCT TCG G	5′-end-labeled digoxigenin probe; ITS2 region of *C. neoformans*

表四 以免疫酵素測試法分析種別專一性核酸探針偵測病原真菌之特異性

種別	A650 平均值						
	CA	CG	CP	CK	CT	GU	CN
C. albicans	2.487	0.083	0.072	0.055	0.094	0.076	0.063
C. glabrata	0.152	1.845	0.075	0.049	0.073	0.078	0.062
C. parapsilosis	0.083	0.070	2.685	0.053	0.077	0.120	0.061
C. krusei	0.086	0.075	0.076	0.874	0.081	0.092	0.066
C. tropicalis	0.087	0.070	0.074	0.047	2.904	0.076	0.081
C. guilliermondii	0.079	0.054	0.053	0.057	0.055	1.507	0.047
C. neoformans	0.071	0.056	0.056	0.048	0.055	0.058	1.216

表五 用 C. albicans 全細胞評估 PCR-EIA 的敏感性

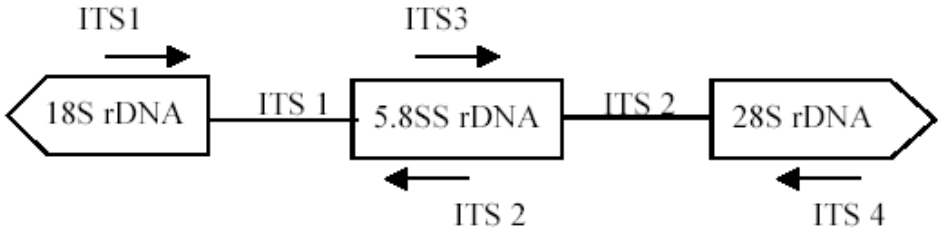
每50 µl 樣本所含細胞數	用 *C. albicans* 專一性探針CA的A_{650} 值
1000	1.301
100	0.919
10	0.273
1	0.072
＜1	0.070

臨界值（cutoff Value）爲陰性對照平均光密度值(OD)的 0.2 倍＝0.260

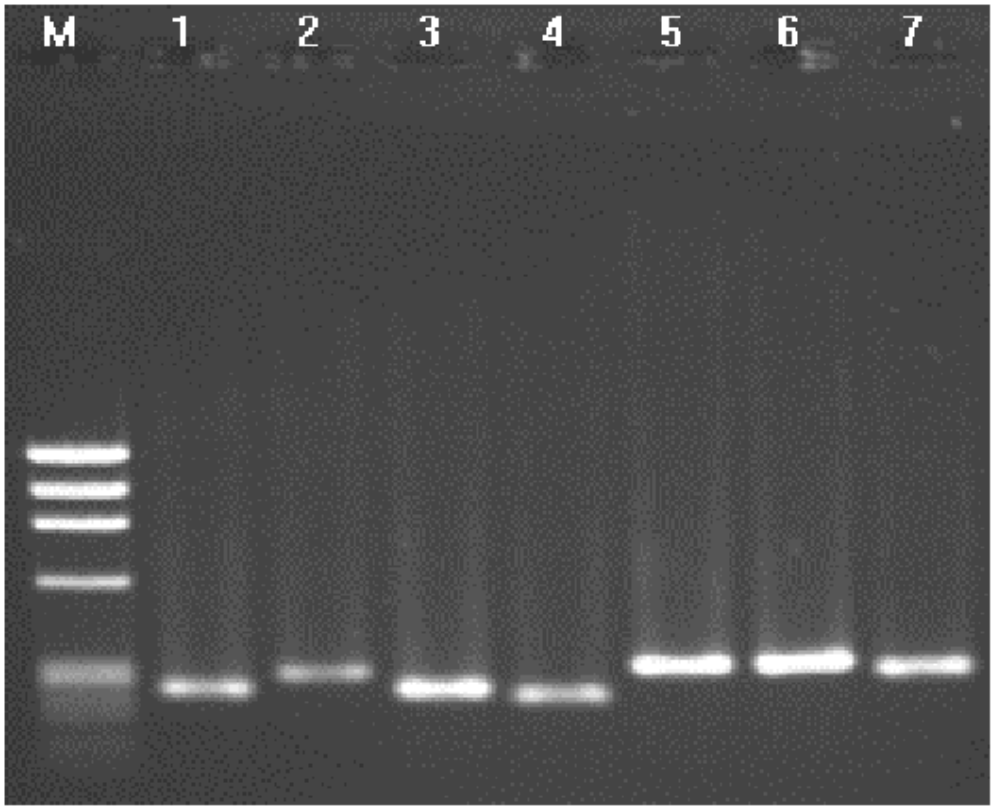
表六 用 C. albicans 萃取 DNA 評估 PCR-EIA 的敏感性

每50 µl 樣本所含DNA量	用 *C. albicans* 專一性探針 CA的A_{650} 值
1 ng	1.0315
100 pg	0.954
10 pg	0.6775
1 pg	0.313
100 fg	0.1
10 fg	0.1215
1 fg	0.082

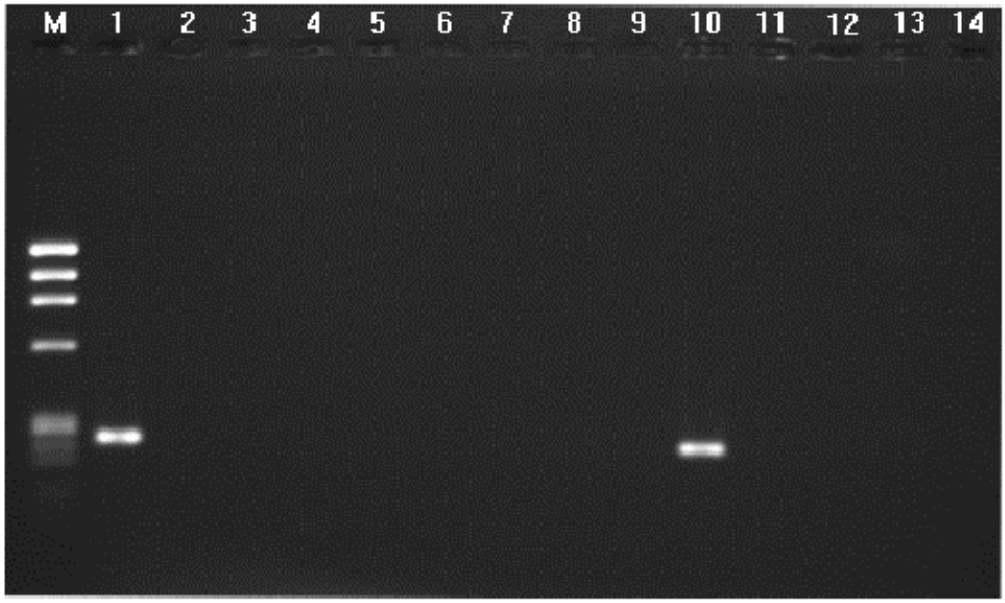
臨界值（cutoff Value）爲陰性對照平均光密度值(OD)的 0.2 倍＝0.248



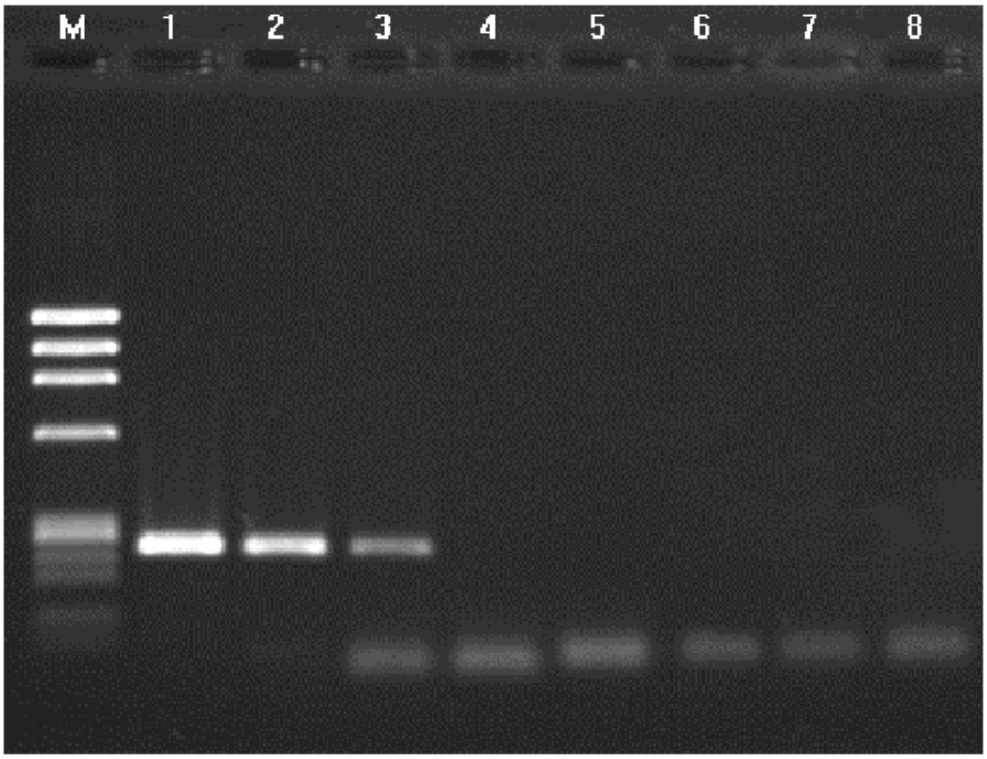
圖一 泛真菌引子(pan-fungal primers) ITS1-ITS4 及種別專一性引子在真菌 rDNA 之區域。



圖二 利用種別專一性引子(CAL, CGL,CPA, CKR, CTR, CGU, CN)針對真菌 DNA 做 PCR。Lanes M：分子量標記(HaeIII-digested fX174 replicative-form DNA); Lanes 1 至 7 分別爲 PCR 產物 C. albicans, primer CAL; C. glabrata , primer CGL, C. parapsilosis, primer CPA; C. kruseis, primer CKR; C. tropicalis, primer CKR; C. guilliermondii,primer CGU; C. neoformans primer CN.



圖三 以 PCR-電泳凝膠分析測試引子的專一性。Lanes 1 到 7 使用 CAL 引子對分別增幅 C. albicans, C. glabrata, C. parapsilosis , C. kursei , C. tropicalis , C. guilliermondii , C. neoformans 標準菌株的 DNA。 Lanes 8 到 14 使用 CAP 引子對分別增幅 C. albicans, C. glabrata, C. parapsilosis , C. kursei , C. tropicalis , C. guilliermondii , C. neoformans 標準菌株的 DNA。 Lanes M, 分子量標記(HaeIII-digested fX174 replicative-form DNA)



圖四 以 C. albicans (ATCC14053)調成不同菌液濃度測試 PCR 之敏感度，primer ITS3 與 CAL 測試：lanes 1 到 8 分別爲每毫升 1x106, 1x105, 1x104, 1x103, 1x102, 10, 1 CFU, Lanes M, 分子量標記 (HaeIII-digested fX174 replicative-form DNA; 片段大小以鹼基對表示)