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行政院衛生署疾病管制局九十三年度科技研究發展計畫

病原真菌之快速檢驗及分子流行病學群體計畫 I
—發展快速種別鑑定方法及分子分型技術

研 究 報 告

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＊本研究報告僅供參考，不代表衛生署疾病管制局意見＊

目　　錄

計畫摘要：

（1）中文摘要：

中文關鍵詞：病原真菌、快速分子檢驗、種別鑑定、分子分型

近年來因人類免疫不全病毒HIV之感染及先進醫療等因素、導致免疫功能不全之個體增加，助長了病原真菌感染之趨勢，進而成為嚴重的公衛問題。欲研擬防治對策，有待發展快速分子種別鑑定方法以助於精確投藥診療，及發展分子分型釐清病原真菌傳播途徑。

在快速檢驗方面，過去我們已發展出快速鑑定六種念珠菌及新型隱球菌種別的 PCR, PCR-EIA 及 real-time PCR 技術，今年度則承續過去的努力發展鑑別念珠菌 fluconazole 抗藥性種別的 PCR-EIA，並將這些方法應用於血液培養檢體上。我們針對臨床上對 fluconazole 具敏感性的 *Candida albicans*, *Candida dubliniensis*, *Candida parapsilosis*, *Candida tropicalis* 的四種念珠菌，對 fluconazole 易產生抗藥性的 *Candida glabrata*，對 fluconazole 完全具抗藥性的 *Candida krusei* 分別設計三組引子。本抗藥性種別鑑定方法專一性高，靈敏度可達 10fg/100μl 以下。以臨床及標準菌株及血液培養檢體測試結果，與綜合 germ tube , Viteck 及 API20C 的生理生化鑑定法結果符合度達 100％。本方法應能有助於臨床診斷、投藥之參考。

在分子分型方面，我們共發展出六種分子分型方法包括三種 PFGE 分型法（PFGE-karyotyping，PFGE-*Sfi*I 與-*Bss*HII）、rep-PCR、MLST 及 AFLP 分型方法，並應用這些分型方法分析從台灣各醫院收集的 *C. albicans* 臨床菌株。PFGE-*Bss*HII 及 MLST 在鑑定 DNA 型態識別力最高，其次是 PFGE- *Sfi*I，再者為 rep-PCR，接著是 AFLP，而 PFGE-karyotyping 識別力最低。結合不同原理的分型方法（如 rep-PCR＋PFGE）可提高分型的鑑別力。這些方法均可鑑定出流病相關菌株，如從相同病人分離出的菌株。而型別為病人專屬和分離部位，地源性與抗藥性無相關。我們也利用 PFGE-karyotyping 和 PFGE- *Sfi*I 分型方法探討 HIV 患者長期移生菌株的分子流行病學。結果顯示，來至同一病人之菌株即使採集分離時間隔很遠，對 fluconazole 的感受性有異，且病原時而移生，時而感染，其親緣關係仍很近。各分型方法經比較後，將所發展最有識別力的分型方法加以標準化，未來可當作疾病爆發研究工具及其他分型的比較基礎。此研究獲得的資料也將有助於於建立真菌病原體中心基因資料庫，並提供國內及國際性真菌基因型比較的平台。

本研究論文成果甚為豐碩，二篇即將刊登於SCI期刊，一篇已投寄至SCI期刊，一篇已發表於國內期刊論文，多篇正在撰寫中即將投寄至SCI期刊。

（2）Abstract:

Keywords：pathogenic fungi, rapid molecular diagnosis, species identification, molecular typing.

The advent of HIV epidemic in recent years together with the modern medical practices have resulted in an increase in the size of the population of immunocomprosed individuals. Consequently, the infections caused by pathogenic fungi have increased rapidly and become major public health problem. Rapid and accurate identification of the pathogenic fungi to species level is critical for timely and proper treatment. Molecular typing is important in elucidating the transmission route. Both rapid diagnosis and molecular typing studies are important for consolidating control strategy.

In our previous research work, we have successfully developed rapid molecular diagnosis methods such as PCR, PCR-EIA and real-time PCR for rapid species identification of 6 *Candida spp.* and *Cryptococcus neoformans*. As a continuation of our previous effort, we have further developed a PCR-EIA method to differentiate 7 clinically frequently encountered *Candida spp.* based on their resistance characteristics to fluconazole. 3 set of primers were designed: one set is for fluconazole-susceptible species including *Candida albicans*, *Candida dubliniensis*, *Candida parapsilosis*, *Candida tropicalis*, one set is for *Candida glabrata* which developed resistance to fluconazole quite easily, one set is for *Candida krusei* which is innately resitance to fluconazole. Specificty of this methods were 100%. Sensitivity was less than 10fg/100μl of the fungal DNA. The identification results matched results of the phenotypical

identification method employing germ tube, Viteck and API20C methods. We have also applied these molecular diagnosis methods on blood culture specimen. The method developed will be beneficial not only for clinical diagnosis but also for therapy regimen.

Reguarding molecular typing, we have developed 6 molecular typing methods, including 3 PFGE-based typing methods (PFGE-karyotyping, PFGE of *Sfi*I- and *Bss*HII), rep-PCR, MLST and AFLP methods and applied these methods to investigate the genetic profiles of *Candida albicans* clinical isolates. All these methods were able to identify clonal related isolates from the same patients. PFGE-*Bss*HII and MLST exhibited the highest discriminatory power, followed by PFGE-*Sfi*I, then by rep-PCR, then by AFLP, while PFGE-karyotyping demonstrated the lowest discriminatory power. High discriminatory power can also be achieved with a combination of typing methods with different typing mechanisms, such as rep-PCR supplementary to PFGE-based typing methods. The results also showed that the DNA type of each isolate was patient-specific and not associated with source of isolation, geographical origin or antifungal resistance. We have also applied both PFGE-karyotyping and PFGE-*Sfi* I analysis methods to study the molecular epidemiology of long-term colonization of *C. albicans* strains from HIV-infected patients. Clonality analysis demonstrated that isolates from same patient sampled over a rather long span of time, despite of different susceptibility to fluconazole and reguardless of colonizing/infecting stage were genetically closely related. These methods will be compared and the most discriminatory typing method will be standardized and serve as the tool for

future outbreak investigation and the basis for comparison basis of other typing methods. The data obtained in this study will also contribute to our attempt to establish a central genetic database of fungal pathogens and provide plateform for comparison of domestic as well as international fungal genotypes.

The publication output of this research is quite fruitful: two papers will be published in SCI journals, one paper has been submitted to SCI journal, and one article has been published in Taiwanese peer-reviewed journal, many manuscripts are in preparation and will be submitted to SCI journals.

本文

一、前言：

近二十餘年來，因愛滋病毒的肆虐、癌症、抗生素及類固醇之濫用及器官及骨髓移植、侵襲性療法、重症及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，使得真菌感染之盛行率大幅攀升[1,2]。除上述機緣性感染之案例外，肇因於都市發展、人口遷移及自然災害等因素[3]，病原真菌對於健康個體之威脅性亦與日俱增[4]。而新的致病原崛起[4]及抗藥性菌株的浮現更增加問題的棘手性。以美國為例，自1989年起念珠菌 *Candida spp.* 感染已躍居院內血流性感染的第四位[5]。其中白色念珠菌*Candida albicans* 佔系統性念珠菌屬的50%且為最具病原性的念珠菌[6]。根據美國院內感染監視系統NNISS之傳染病死亡案例統計亦顯示，真菌性病害的排名已由1980的第十位躍升至1997年的第七位[7]。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[8]。美國曾見報導治療每位念珠菌血症(candidemia)病患花費約在3~4.5萬美元間，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[9]。

在國內的真菌盛行率增加情形也極類似，甚至更為嚴重。自2001年起 *Candida spp.* 屬及其他酵母菌以高居國內醫學中心院內

感染病原第三位, 其中以 *C. albicans* 高居酵母菌感染的榜首。在台灣某教學醫院念珠菌高居院內感染致病菌的榜首[10]。依據該醫院統計1981至1993年院內感染真菌共增加了27倍。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%。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[11]。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[12,13]。

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。*Fusarium*, *Mucor*白黴菌和*Aspergillus spp.*麴菌等所造成的全身性感染，死亡率甚至高達100% [14]。如此高的死亡率與目前在診斷困難有相當的關係。顯示真菌感染，在今日醫療中不可輕忽的地位。

抗真菌藥物仍有許多改善的空間[15,16]。這是因為早期診斷不易，延誤投藥時機。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。近來多種具有不同作用範疇的替代藥物如voriconazole, posaconazole, echinocandin, caspofungin [17]的上市，使

得醫師在針對不同種別真菌用藥時有更多選擇。因此早期診斷以精確篩選出需要接受抗真菌藥物治療的病人十分必要，此舉可望有助於精確投藥，節約昂貴藥物的治療費用，並避免產生副作用。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，不同種別的真菌對藥物的抗性不一[18]，更增加問題的棘手性。舉例如amphotericin B, flucytosine, itraconazole, ravuconazole及voriconazole對不同黴菌的抑菌濃度也有顯著不同[19]。如*Candida lusitaniae*對 amphotericin B容易產生抗藥性[20]。對 fluconazole，*Candida krusei*完全具抗性，*C. glabrata*則較其他*Candida*菌種具感受性 [21]。*C. tropicalis*非常容易產生fluconazole的抗藥性[22]。精確鑑定出種別，對醫師選擇藥劑種類及劑量，避免抗藥性的產生，將益形重要。因此有必要建立快速抗藥性鑑定方法及這些菌株的抗藥性及臨床相關資料。如此希望能對全國病原真菌之抗藥性分佈有初步的瞭解。

早期及正確的診斷有助於病害診療及防治[23,24]。病原真菌在診斷上頗為困難。且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，高敏感度的偵測甚為重要。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.* 如*C. albicans*, *C. tropicalis*, *C. parapsilosis*, 和 *C. glabrata* 以及*Cryptococcus neoformans*, *Aspergillus spp.* , *A. fumigatus*及*A. niger*等。許多較罕見的真菌在近年來被發現引發感染。病原真菌之傳統鑑別診斷方法一般結合培養法如用SDA、BHI、CHROMagar培養及染色法如用KOH、India Ink[25]，觀察型態為主。然培養法費時久、過程複雜、需累積的經驗。且敏感度sensitivity不夠高。生化生理檢測法如用API ID32C (bioMérieux Inc., France)、VitekII、Rapid檢測法等商業化快速鑑定的套組[26]，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[27]。近來，一些非培養分析法如應用免疫檢測的樹脂凝集試驗法(Latex Agglutination, LA) [28]、免疫擴散法(Immunodiffusion, ID)、酵素免疫反應(Enzyme immunoassay, EIA) [29]、免疫螢光染色(immuno florescence, IF) [30]、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[31]。例如，利用LA檢測Cryptoccocus neoformans抗原，以三明治EIA[32], IF[33]偵測麴菌和念珠菌抗原、或利用EIA、ID或CF測blastomycosis, coccidioidomycosis, paracoccidioidomycosis及

histoplasmosis等之抗體力價[34]。然而，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以達能快速、精確及敏感偵測之目的[35]。晚近，核酸檢測技術之崛起提供快速、精確及敏感鑑別之利器[23]。在疾病診斷及種別鑑定上，聚合酶連鎖反應(polymerase chain reaction, PCR)最為常見；PCR具有快速鑑別pg微量或單一細胞之靈敏度，而改良過後的套疊式PCR (nested-PCR)、半套疊式PCR (Semi-nested PCR)[36]以內外兩對引子，可增強鑑定及鑑別度。以PCR配合酵素免疫檢測法EIA或RFLP技術[37,38] 以及核酸雜合法[39,40]檢測真菌已有不少報告，在Candida方面，已有先以PCR增幅ITS2區段，再以專一性molecular beacons探針鑑定Candida dubliniensis[41]之文獻。此外如應用在*Aspergillus spp*[42], *Candida spp.* [43,44], *Cryptococcus neoformans*[45], *Penicillium marneffei*及重要的雙型性酵母菌[46]等之鑑別。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)，用來檢測*Pneumocystis carinii*肺炎[47]、*C. albicans*、*C. neoformans*等病原。及時PCR偵測法具有快速(45min-2hr)、敏感度更高、可定量及避免污染等優點。吾人日前發展出可鑑定7種臨

床上常見真菌種別之PCR-EIA[48]。也發展以Light-Cycler Real-time PCR及時偵測7種臨床上常見真菌病原之檢驗系統[49]。晚近在2000年，日本Notomi等人研發出一種名為Loop-mediated Isothermal Amplification(LAMP)的新型核酸增幅技術[50]，藉由精密的引子及反應設計大幅提昇了鑑別力，並簡化了反應的過程，可不需要精密度高的快速溫度循環儀或檢測機器，適合田間篩檢、現場(point-of-care)或病床邊測試、初級照護機構、田野分子流行病學調查或大規模檢疫工作之用。目前LAMP在日本已研發出SARS[51]、退伍軍人症[52]等檢驗試劑，這兩種病原的傳播速度快，波及範圍廣，而LAMP的簡便、快速和精確可加快醫療措施及防疫的啟動。

隨著序列分析的重要性增加，有許多研究者以菌種之間序列的核苷酸組成或長度差異來鑑定菌種[53,54]，針對基因標的為ITS2或large subunit ribosomal DNA，可鑑定臨床重要真菌[55,56]。Sugita等已經建立一個ITS序列資料庫來鑑定臨床重要的*Trichosporon spp.*，需時24小時以下[57]。Ninet等發展一套利用28S核酸序列來鑑定皮膚真菌菌種(dermatophytes species)的商業套組；ABI公司則發展出針對真菌的D2 LSU rDNA片段可全自動化分析序列的系統，並經由資料庫比對鑑別種別。定序粒腺體的large subunit ribosomal RNA基因，亦可提供作為除細胞核rDNA外另一比較類緣及區別鑑定之標的

[58]。這些真菌菌株基因序列資料若能更臻完備，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。此領域之進一步應用則是發展核酸陣列晶片，點佈數百至數萬點的探針，且依臨床或防疫需求，除可廣泛涵蓋臨床上常見病原[59]，甚可納入可能變種及人畜共通病原，如此一來，可以極少量檢體做較全面性的檢驗鑑定。國內晶片技術平台經數年研發以來，各種macro-/microarray乃至lab-on-a-chip技術已漸趨成熟，因此發展快速鑑定致病性酵母菌之DNA陣列晶片也是可行之方向。

C. albicans 等伺機性真菌為人類黏膜常規的移生菌。對免疫機制正常的宿主僅偶而會造成嬰兒鵝口瘡及婦女陰部感染。在免疫缺損的病患卻可能造成高死亡率的系統性感染[60]。HIV 患者 *C. albicans* 帶原量及引發鵝口瘡之機率在 CD4+ 數量低下時明顯升高[61]。不明的口腔念珠菌病向來被視為感染了 AIDS 或 HIV-病人免疫缺損惡化的先期指標[62]。HIV 病人因抗藥性菌株引發的口咽念珠菌症已成為主要問題[63]，1/3 的病人晚期有口腔有 *C. albicans* 抗藥性菌株的出現[64]。依據台北市立性病防治所 207 位 HIV 感染患者口腔表徵之發生率，發現口腔念珠菌病為 12.1%。其中仍以 *C. albicans* 最為常見，而非 albicans 的念珠菌感染與 CD4 lymphocyte 低下及 azole 療程有

關，這些非 albicans 的念珠菌也較易發展出抗藥性且常與 *C. albicans* 混合感染，因此在治療投藥時應加以注意[65]。

念珠菌為常見的院內感染病原，多數感染案例之流行病學調查指向病人本身共生菌叢伺機感染所致，而非院內交叉感染所致，近來研究卻顯示外源感染(exogenous)如源自醫護人員、器械、環境之可能性[66]。各種病原真菌分子分型(molecular typing)的方法應運而出，有助於種別鑑定、菌種共生/感染、傳播途徑[67]、防禦工作[68]、療效追蹤、菌株消長[69]、抗藥機轉、多樣性分析[70]等臨床治療諸問題之闡明。例如，將基因組用酵素*Eco*RI, *Hinf*I和*Msp*I做酶切片段長度多型性(**RFLP**; restriction fragment length polymorphism)[71]或RFLP結合探針雜交被應用於反映*C. albicans*菌株間類緣關係以追蹤菌株長期的微演化和追溯感染源[72]並決定地理的趨適性和抗藥性的特別表現[73,74]。**EK**是利用脈衝式電泳，讓染色體DNA分離開來。Pulse-Field Gel Electrophoresis (**PFGE**)則為將真菌染色體DNA以切點少的限制酶如*Sfi*I [75]、*Sma*I、*Not*I及*Bss*HII [76]水解後，將片段用脈衝式電泳得到特異性圖譜[77]。這些PFGE分子分型方法被廣泛用來做流病爆發追蹤傳播途徑之調查，PFGE識別力及再現性均佳，堪稱為是流行病學分子分型分析的黃金標準。然而PFGE具有通量低(low throughput)、不易標準化、比對不易等缺點。全部基因組的PCR-fingerprinting則包括

Randomly amplified polymorphic DNA (**RAPD**) [78]或者是amplified fragment length polymorphism (**AFLP**)[79]，二者除分型外可用於建立菌株相關性及鑑別菌株特殊標記片段。RAPD利用隨意引子以非嚴苛(non-stringent)條件增幅所得圖譜區分型別，方法雖簡便，但再現性不高。AFLP原理為用二種限制酶(如*Eco*RI和*Mse*I)切割DNA後，再以雙股ＤＮＡ接合子adaptor黏合於DNA片段二端。接著以接合子序列當作引子做PCR，其中一條引子以螢光標幟以得片段圖譜。為減少分析片段圖譜複雜度，可於引子的3'端多加1～3個核苷酸，以特定增幅某部分片段，簡化分析。AFLP之優點為所需DNA量微，通量高，且利用嚴苛的PCR條件故再現性相當高。核酸定序分析應是最客觀理想的分子分型工具，近來自動化定序技術越臻成熟，成本越趨合理，使得定序用於例行性分型工作可行性增高。數年前，若干英法學者倡議建立milti-locus sequence typing (**MLST)** 藉由鑑定序列上的核苷酸多型性做為病原真菌國際化網路上比對的平台 http://www.mlst.net。MLST原理為針對6-8個約500bp的持家基因(house-keeping genes)定序，每一條不同的序列皆被歸於一特定的alleles。每株菌株則依其這些特定持家基因locus上alleles的組合樣式形成其特定的序列樣式(sequence type, ST) 以茲比較。MLST具有穩定、鑑別力高[80]，利於實驗室間資料交換比對等優點[81]，並被成功應

用於探討*C. albicans*的院內感染流行病學及評估全球菌株之多樣性及菌株間類緣關係[82]。以上技術所針對的sequence或片段經常分佈於所有genome，反覆序列指紋法如rep-PCR [83]與Multilocus microsatellite genes分析則應用在基因組上某些特定位子散置出現重複性的DNA。如針對高變化頻率的satellite DNA上的micro-及minisatellite所發展的**VNTR** (variable number of tandem repeats)或**MLVA** (multiple-locus VNTR)分型技術則被用來探討*C. albicans*的演化和族群遺傳結構[84,85]。綜言之，適當分子分型工具的挑選應視生物特性和探討的目的而定，理想上必須具再現性、識別力、容易使用，且需朝向高通量、可數位輸出、網路化、標準化與建立資料庫[86]。

分子分型技術亦被廣泛應用於探討HIV病人上*C. albicans*菌株之分子流行病學。曾見報告感染HIV病人的感染性菌株與共生菌株不同[87]。然而也有研究觀察健康帶原者的分離株與HIV感染病人的菌株相類似[88]。曾有*C. albicans*抗藥性菌株在家族成員間[89]或經由伴侶口腔接觸而平行傳播[90]的報告，內源性感染之案例亦見報導[91]。HIV病人菌株感受性的改變可能由於抗藥性菌株的平行傳播或內源性菌株產生突變。以EK及用27A重複性序列雜和，針對接受fluconazole治療HIV患者口腔所分離之*C. albicans*菌株做型別區分。結果顯示HIV病患口腔具複雜菌相，但各菌株仍具相關性，

且有些菌株在治療過程會產生fluconazole抗藥性[92]。27A探針雜和技術鑑別菌株，發現型別與地理區及抗藥性無關，亦反駁HIV患者所分離fluconazole抗藥性菌株具同一起源之說法[93]。女性HIV病患常同時感染口腔及陰道念珠菌病，以RFLP及RAPD型別分析卻顯示，不同部位菌株的型別不同[94]，流行病學特性亦有差異，口腔念珠菌病好發於CD4數目低下之患者，陰道念珠菌否，這或許反映出口腔及陰道二部位黏膜免疫機制的不同[95]。

本計畫之研究成果甚為豐碩，較具體且已實際發表的可大分為三項：

(一)、收集全省代表性菌株，精確鑑定種別並測定這些菌株的抗藥性(與國衛院合作)。發展有助於臨床醫生投藥的PCR-EIA快速鑑定方法並評估新型LAMP快速鑑定法。

(二)、應用各種分型技術，認識全國真菌之分子流行病學概況。比較各分子分型法識別能力，以及查明是否不同特性（例如抗藥性、地源性、分離來源、是否院內感染）可歸類為某種台灣特定的型別。以瞭解各方法的實際適用性並研究從台灣各醫院所分離出來的*C. albicans*臨床株之型別。

(三)、應用各種分型技術，探討感染HIV病患*C. albicans*之分子流

行病學。

所發展的PCR-EIA快速鑑定方法希望能有助於醫生臨床判斷投藥。至於分子分型方面，目標在於比較後把最有識別力的分型方法標準化，這可當作將來疾病爆發研究工具及其他分型的比較基礎。這些菌株的分子型別資料也將建立於真菌病原體中心基因資料庫，且提供國內及國際真菌基因型比較的平台。

二、材料與方法：

(一)、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 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)。每一株臨床菌株包含的資訊有 fluconazol 抗藥性測試、地域來源和由身體何部位分離，還包含是否為院內感染 [96]。主治醫生根據美國疾病管制局制訂標準決定是否為院內感染 [97]。每次院內感染事件僅取一株代表性菌株。*C. albicans* 分離株的 fluconazol 抗藥性 MICs 測試用 microdilution broth 的方法，根據 NCCLS 公布文件 M27-A[98 99]。

四種分型方法比較共使用 53 株臨床性 *C. albicans* (菌株特性詳見表五)。其中 42 株臨床菌株來自 TSARY I 計畫收集的菌株。剩下的 11 株分離株(CDC-F003000393~ CDC-F003000396, CDC-F003000399~ CDC-F003000402, CDC-F003000418~ CDC-F003000420)，為在 2001 年至 2002 年間，從同一間醫院的 3 位愛滋病感染病人口腔中分離出的菌株。

以 PFGE-Electrokaryotyping 及 PFGE-*Sfi*I 探討來自 HIV 病人念珠菌的分子流行病學所使用的 21 株臨床 *C. albicans* 菌株（CDC- F003000393~ CDC-F003000396, CDC-F003000399~ CDC-F003000402, CDC-F003000418~ CDC-F003000420）(菌株特性詳見表九)。為在 2001 年至 2002 年間，從同一間醫院的 3 位愛滋病感染病人(病人#1~#3)口腔中分離出的菌株。我們挑選三

名於台灣大學附設醫院感染科門診就醫的男性 HIV 感染患者。收集病人基本及臨床資料包括:年齡、性別、最近的 CD4+ 淋巴球數目及最近是否接受抗反轉錄病毒治療、抗真菌用藥治療歷史和發生口腔或口咽念珠菌症與否。經病人非正式口頭同意後，從這 3 位 HIV 患者以乾海綿採檢棒做口腔採檢收集檢體做培養。病人#1 和#2 分別於 1999, 2001, 和 2002 年採檢。病人 #3 則於 2001 和 2002 年採檢。每支採檢棒至少挑取二株獨立的菌落作後續進一步分析。

以 rep-PCR 探討來自 HIV 病人念珠菌的分子流行病學所使用的 91 株臨床 *C. albicans* 菌株（45 株來自 HIV 病人與 46 株來自非 HIV 病人）(菌株特性詳見表九)。

發展 MLST 分型方法共計使用 28 株 *C. albicans* 臨床菌株和 2 株標準菌株(菌株特性詳見表八)。其中的 28 株臨床菌株可大分為 3 組：第一組包含 7 株 1999 和 2002 年間從 3 位 HIV 病人來的口腔分離株(CDC-F003000393, CDC-F003000397, CDC-F003000402, CDC-F003000404, CDC-F003000406, CDC-F003000416, CDC-F003000419) ；第二組包含 6 株從非 HIV 病人來的臨床株。第三組包含 15 株 TSARY 計畫從全省醫院收集的菌株，且每一群流病感染僅取一株代表菌株，故這 15 株菌株間皆為流病不相關的菌株。

發展 AFLP 分型方法共計使用 56 株 *C. albicans* 臨床菌株和 xx 株標準菌株(菌株特性詳見表 xx)。其中的 28 株臨床菌株可大分為 3 組：第一組包含 7 株 1999 和 2002 年間從 3 位 HIV 病人來的口腔分離株(CDC-F003000393, CDC-F003000397, CDC-F003000402, CDC-F003000404, CDC-F003000406, CDC-F003000416, CDC-F003000419) ；第二組包含 6 株從非 HIV 病人來的臨床株。第三組包含 15 株 TSARY 計畫從全省醫院收集的菌株，由於收集時每一群流病感染僅取一株代表菌株，故這 15 株菌株間皆為流病不相關的菌株。

(三)、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。

(四)、DNA 萃取：

真菌分離株培養後用 PUREGENE DNA Purification Kit (Gentra, Minneapolis, Minnesota, USA)萃取 DNA 。真菌在 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。

(五)、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。

(六)、PCR 引子之設計：

參考文獻並從 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，設計各真菌之引子及核酸探針。

(七)、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 50μl，內含 1μl 待測 DNA，25μl 2 X PCR 緩衝液（MBI Fermentas 2X PCR Master Mix），0.5μM 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-200MJ research。試劑製備反應全程均有依據 Kwok 及 Higuchi 的預防污染建議[100]。

(八)、PFGE-染色體檢查：

先取菌種塗佈在 SDA 培養基上，置於 37℃培養 48 小時後，刮取菌落溶至細胞懸浮緩衝液中（cell suspension buffer）（100mM Tris-HCl, pH 8.0; 100 mM EDTA, pH 8.0），測其菌量濃度 O.D.600 值為 1.5。轉速 3000g 離心 5 分鐘，吸去上清液後加入 500μ 細胞懸浮緩衝液，再加入 100μl 細胞溶解酵素（lyticase）（Sigma, 1250 unit/ml in 50% glycerol 和 0.01 M N3PO4），放置 37℃下反應 30 分鐘。之後，加入 600μl 含 1%（w/v）瓊脂（Seakem Gold agarose, BroWhittaker Molecular Applications）的 TE 緩衝溶液（10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0）混合，再到入製膠模中，放置室溫約五分鐘讓其凝固。將凝固的膠片轉放置含 5 ml 細胞溶解緩衝溶液（100 mM Tris-HCl, pH 8.0, 0.45 M EDTA, pH 8.0, 1% N- lauroylsarcosine, 1 mg/ml proteinase K）的 50 ml 離心管中，放置 50℃水浴槽搖晃反應一個晚上後，用 50°C 滅菌水清洗 15 分鐘，連續兩次，再用 50°C TE 緩衝溶液清洗 10 分鐘，最後放置 TE 緩衝溶液保存。將切好的膠片與配製好的 0.8%瓊脂膠放入 0.5x TBE 緩衝液（50 mM Tris, 45 mM Boric acid, 0.5 mM EDTA）中使用 Biometra Rotaphor®儀器跑 66 小時電泳，其變換秒數 60-700 秒，角度 120°，電壓 120-90V。將瓊脂膠放入

EtBr 溶液中染色染色 15 分鐘後，蒸餾水退染。DNA 圖譜用 IS-1000 數位影像系統(Alpha Innotech Corporation)照相存檔。

(九)、PFGE-SfiI 和-BssHII 限制酶切片段：

將膠片（plug）切成 2mm 寬的薄片。*Sfi*I 酶切前步驟為將切好的膠薄片放入 2μl BSA （New England BioLabs, Beverly, MA, USA）與 200μl2 號緩衝溶液（50mM NaCl, 10mM Tris-HCl, 10mM MgCl2, 1mM DTT）中，50℃反應 1 小時。之後將膠薄片轉至含 2μl BSA、20 個單位的 *Sfi*I 酵素與 200μl2 號緩衝溶液中，50℃反應一晚。*Bss*HII 酶切前步驟為將切好的膠薄片放入 200μl3 號緩衝溶液（100mM NaCl, 50mM Tris-HCl, 10mM MgCl2, 1mM DTT）（New England BioLabs, Beverly, MA, USA）中，50℃反應 1 小時。之後將膠薄片轉至含 4 個單位的 *Bss*HII 酵素與 200μl3 號緩衝溶液中，放置 50℃反應一晚。將酶切過的膠薄片與配製好的 0.8%瓊脂膠放入 0.5x TBE 緩衝液(50 mM Tris, 45 mM Boric acid, 0.5 mM EDTA)以 Biometra Rotaphor®儀器跑 36 小時電泳，其變換秒數 60-700 秒，角度 120°，電壓 120-90V。電泳完後，將瓊脂膠放入含溴化乙錠溶液中染色染色 15 分鐘後，再用蒸餾水退染。DNA 片段圖譜用 IS-1000 數位影像系統（Alpha Innotech Corporation）照相存檔。

(十)、rep-PCR：

DNA 的純化見(三)。Rep-PCR 反應依前人發表稍做修改使用引子序列為 Ca-21 5’-CATCTGTGGTGGAAAGTTAAC-3’ 和 Ca-22 5’-ATAATGCTCAAA-GGTGGTAAG-3’ (19)。反應混合物組成有 10 mM Tris-HCl (pH=9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM dNTP mix, 50 pmol 引子和 0.5 個單位的 Taq polymerase。使用 Tpersonal thermocycler （Biometra）儀器進行增幅反應，起始溫度是 95°C 五分鐘，之後三個步驟為 94℃讓 DNA 變性 1 分鐘，42℃黏結 1 分鐘，72℃擴大 2 分鐘做 40 個循環，最後 72℃擴大 5 分鐘。PCR 產物與 1.5%瓊脂膠放入 1 倍 TBE 緩衝液（Seakem LE agarose, Cambrex）

中，用 100 V cm-1 跑 50 分鐘電泳。DNA 標準標記有 11 條 100 到 3000bp 的片段，可確定 PCR 產物 DNA 的大小。電泳跑完後，將膠片放入含溴化乙錠溶液中染色 15 分鐘，再用蒸餾水退染。

(十一)、DNA 片段分析：

利用軟體 Bionumerics software version 3.0（Applied Maths, Kortrijk, Belgium）畫樹狀圖分析。指紋法的特色就在於比較每條 DNA 片段的有無。片段範圍受參考標記（reference marker）大小校正。片段分析首先靠軟體的自動收尋片段功能尋找，然後用檢查確定片段確切位子，而酶切不完全的微弱片段用手動方式將其去除。用不同濃度的酵素去切，看其不完全的中間片段重複情形，確定酶切不完全的 DNA 片段位子。位子差異範圍在 1%可忍受，而最佳化是設在 3%。使用擲骰子係數（Dice Coefficient）去分析片段相似度（SAB），用算數平均（UPGMA）作一連串的分析。當分離株相似度小於 95%時，被認為是不同的[101]。

(十二)、鑑別力的計算：

四種分型法的個別識別力指數（discriminatory index, DI）由辛普森多樣性指數（Simpson's Index）決定[102]。測試族群中兩個不相干的種被放入不同型族群中，DI 數值如果是 1.0，表示此種與此族群可區分；相反的，DI 值為 0.0，表示此種與此族群為同一種。

(十三)、EIA：

PCR-EIA 設計使用之引子及探針序列詳見表一。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, 和 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 (Biotek 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 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。或抽取 DNA，將抽取的 DNA 從 1ng 到 1fg 依序做 10 倍序列稀釋。

(十四)、增幅片段長度多型性分析(amplified fragment length polymorphism, AFLP)

a. 限制酶切及以接合子接合:

取 10^7 CFU 的真菌細胞並抽取 DNA。取 100ng 的染色體 DNA 並加入 5U 的 *Eco*RI 和 1U 的 *Mse*I (New England Biolabs, Beverly, Mass.) 於酵素緩衝液(restriction buffer)內進行酶切，反應總體積為 10 μl 。酶切反應 37℃下進行 2 小時。限制酵素切後， 取 10μl 的酶切產物移置新反應管，內含已加入的 0.5μl 1mg/ml BSA, 1μl 0.5M NaCl, 2 pmol 的 *Eco*RI 接合子, 20 pmol of the *Mse*I 接合子, 80 U of T4 DNA ligase, 2 μl of a 10X T4 ligase buffer (United States Biochemical, Cleveland, Ohio)。反應總體積為 20 μl. 接合反應於 37℃下進行 2 小時。AFLP 所用接合子和引子如下：

接合子或引子		序列
Adaptor	*Eco*RI adaptor	5'-CTCGTAGACTGCGTACC-3'
		3'-CATCTGACGCATGGTTAA-5'
	*Mse*I adaptor	5'-GACGATGAGTCCTGAG-3'
		3'-CTACTCAGGACTCAT-5'

1st PCR Primers	*Eco*RI-A	5'-GACTGCGTACCAATTC**A**-3'
	*Mse*I	5'-GATGAGTCCTGAGTAA-3'
2nd PCR Primers	*JOE-Eco*RI-AA	JOE -5'-CAATTC**AA**-3'
	*Mse*I-C	5'-GATGAGTCCTGAGTAA**C**-3'

b. 前篩選PCR和篩選PCR：

所得反應混合物以水稀釋十倍。使用包含 AFLP primers, core mix, 和 internal size standard 的 AFLP 套組(Applied Biosystems Foster city, CA.)。前篩選 PCR 的 core sequences 為不含 nucleotide extensions 的引子。PCR 增幅反應總體積為 10 µl 內含 2µl 的 10 倍稀釋的接合反應產物, 0.3µl 的 *Eco*RI core sequence, 0.3µl 的 *Mse*I core sequence, 和 7.4µl 的 AFLP amplification core mix。反應物進行 PCR 增幅 (Whatman Biometra T3000 thermocycler machine)。增幅溫度條件： 94°C 5min, 20 次循環的 94°C 30 s 和 65°C 30 s, 最後延長反應 72°C 1 min。再接著最後延長反應 72°C 及 10 min。

接著的後增幅反應則是使用 *Eco*RI-AA-JOE 和 *Mse*I-C 引子(表 2) 增幅溫度條件： 94°C 5min, 12 次循環的 94°C 30 s 和 65°C 30 s, 黏合反應每循環降 1°C，最後延長反應 72°C 1 min。再接著最後延長反應 72°C 1 min 和 23 次循環的 94°C 30 s, 56°C 30 s, 和最後延長反應 72°C 1 min。再接著最後延長反應 72°C 及 10 min。

進行毛細管電泳之前處理則是將 0.5µl 的篩選 PCR 產物添加於 10µl 的混合液，混合液包括 1:40 的 GeneScan-500 6-carboxy-X-rhodanine [ROX] labeled 和 deionized formamide 做為內部標準品。混合液加熱 94°C 3 min, 接著置於冰上冷卻。

c. AFLP片段分離偵測及資料分析:

AFLP 增幅產物以 ABI3100 avant 全自動定序分析儀分析。並以 Bionumerics, 版本 4.0 軟體分析圖譜(Applied Maths) 使用 Pearson 相關當作相似度係數並結合 UPGMA 做樹狀圖群組分析。

(十五)、multilocus sequence typing, MLST

a. 選擇loci:

選擇六個 housekeeping loci 分別為 CaACC1、CaVPS13、CaGLN4、CaADP1、CaRPN2 及 CaSYA1(序列資訊詳見表六)，而每個 locus 使用 primer 經 PCR 反應放大至 500-700bp 之片段。

b. PCR反應及定序

PCR 反應容積為 50μl，內含有 50ng DNA、每種 primer 50pmol、1.25U Taq DNA polymerase、5μl 的 10X buffer 及 200μM deoxynucleoside triphosphate。PCR 反應條件為 95℃, 5min denature，接著進行 30 cycle 93℃, 30S→55℃, 1min→72℃, 1min 的反應，最後為 72℃, 4min 的反應。定序反應使用 ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems)，反應之分析使用 ABI 3100 avant PE Applied Biosystems 儀器。

c. Sequence分析

所得到的 sequence 經過 alignment 的分析處理後貼於 Bionumericus 分析軟體中比較個菌株的類緣關係，並且上 http://www.mlst.net 網站比對屬於該資料庫中的何種 type。

三、結果：

(一)、進行種別及抗藥性鑑定、發展有助於臨床醫生投藥的PCR-EIA快速鑑定方法並評估新型LAMP快速鑑定法。

a. 台灣地區病原真菌之種別及抗藥性分佈：

與國衛院合作收集全省代表性菌株，精確鑑定種別並測定這些菌株的抗藥性，結果詳見附錄已接受文章(14-18頁)。

b. 發展有助於臨床醫生投藥的PCR-EIA快速鑑定方法：

以標準菌株測試所設計三組抗藥性引子的專一性。先以泛真菌引子ITS1及ITS1進行PCR試驗(圖一)，再以增幅產物進行EIA反應，結果顯示臨床上對fluconazole具敏感性的*Candida albicans*, *Candida dubliniensis*, *Candida parapsilosis*, *Candida tropicalis*的四種念珠菌僅對CFS (*Candida* fluconazol susceptible)引子有反應，對fluconazole易產生抗藥性的*Candida glabrata*僅對CFM (*Candida* fluconazol medium)引子有反應，對fluconazole完全具抗藥性的*Candida krusei*僅對CFR (*Candida* fluconazol resistant)引子有反應 (表二)。本抗藥性種別鑑定方法專一性高達100%，靈敏度可達10fg/100μl以下(圖二)。以臨床分離菌

株(表三)及血瓶培養檢體(表四)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%。此外，混合感染的情形也可被清楚地鑑定出來，例如臨床分離菌株方面*C. parapsilosis*與*C. glabrata* 及 *C. glabrata*與*Candida kruse* 各有一例，*C. albicans*與*C. glabrata* 則有二例(表三)，血瓶培養檢體方面*C. tropicalis*與*C. kruse* 混合感染鑑定出一例(表四)。本方法可將菌株依其對fluconazole不同抗藥性加以區分，又能鑑別混合感染，對涉及抗藥性不同菌株的混合感染尤其具意義，應能有助於臨床診斷及投藥之參考。

c. 評估新型LAMP快速鑑定法：

收集整理文獻資料，並初步評估 LAMP 快速鑑定法之實用性，內容詳見附錄(19-25 頁)。依據其他 LAMP 文獻的電泳圖顯示，成功的 LAMP 反應 DNA 在電泳後應該呈現多層次片段，且從 well 開始就有「拖曳」現象，但是自行設計的 primer 及製作的反應結果，相形之下效能顯的極差。雖然電泳結果也有「拖曳」的情況，但 DNA 的產能不佳，只能說 primer 有用，功效卻無法肯定。另外以相同的實驗方法對真菌 *Candida* 測試 LAMP 的可行性，發現效能比較差，且檢驗

的結果不穩定。

(二)、應用各種分型技術，認識全國真菌之分子流行病學概況。

a. 比較四種分子分型方法及探討台灣地區病原真菌之類緣性：

本研究由 45 位病人分離出的 53 株臨床性 *C. albicans* 菌株，用 PFGE-karyotyping(圖三)、PFGE- *Sfi*I (圖四)、PFGE-*Bss*HII (圖五)與 rep-PCR (圖六)比較分析。所有的分離株用四種分型法分型，最有識別力的是 PFGE-*Bss*HII 與 PFGE-*Sfi*I，分別分出 40 型(DI=0.995)與 35 型(DI=0.985)，來自不同醫院的 42 株分離株中，PFGE-*Bss*HII 方法分出 38 型。rep-PCR 方法分出 31 型(鑑別力，discriminatory index, DI=0.983)。PFGE-karyotyping 扣除 R 染色體外，只有 7 或 8 條 DNA 片段，在 53 株中僅區分出 19 型，為識別能力最差的方法(表五)。不同醫院的 42 株分離株均採自獨立的流病事件，對照之下，HIV 病人長年連續的分離株，可當作族群/流行病學的相關分離株。四種分型方法都可把來自三個 HIV 病人收集到的 11 株分離株歸類為三種分子型，來自同一 HIV 病人分離株各歸屬於一種獨特的基因型。

分析型別與表現型及地理來源相關性之結果顯示每個分離株的 DNA 分型是病人特有的，引起院內感染能力或血流感染的菌株並無特定基因型占優勢。由於本研究收集來自於台灣四處的 18 間醫院的菌株，因此有不同地域代表性，然不同醫院基因型廣泛分散，顯現基因型與醫院/地域性兩者間並無相關性。如圖三~六顯示，抗 fluconazol 菌種 (MIC>64μg/ml)在四種分型法的類緣分析下都無法聚集成群，而且長期連續分離自三個接受 fluconazol 藥物的 HIV 病人個別獨特的基因型。顯示每個菌株的 DNA 型別與個別病人有關而和院內感染與否、分離部位、地理區來源, 或對 fluconazole 的抗藥性等無關。

b. 發展先進MLST分子分型方法：

我們以MLST分型方法分析28株*C. albicans*臨床菌株和2株參考菌株(圖七)，顯示6條基因上共有66個多樣性位置(polymorphic sites)，各基因的基因型數約9~19不等，核甘酸變異數佔各基因的5-14%不等，共產生84種基因型 (genotypes) (表六)。

比較MLST和PFGE-*Bss*HII分型方法(表七)。所有這30株菌株皆能被這二種方法分型。 PFGE-*Bss*HII 能區分出

27個分子型別，鑑別力(DI)為0.900；MLST的鑑別力較高，能區分出29個雙型性序列型別(diploid sequence type, DST)，鑑別力(DI)為0.967。被MLST分型為同一型別菌株僅有CDC-F002000393 (來自N4醫院HIV病人#18)與CDC-F002000393(來自S4醫院病人#4) 二株菌株；這二株以PFGE-*Bss*HII分型亦屬於同一型別。有趣且值得注意的是有二批N4醫院的來自二位病人的分離株(病人#16: DOH86e040, DOH86f007及DOH86f044; 病人#17: DOH86d082, DOH86d090及DOH86f022)，以PFGE-*Bss*HII分型的結果發現DOH86f007及DOH86f044(分別分離自胸腔積液及痰液)菌株和DOH86d090及DOH86f022(分別分離自直腸拭子及肛門拭子)菌株雖各為不同日期採檢，兩兩各屬相同型別。而DOH86e040(分離自直腸拭子) 雖與DOH86f007同一天採檢，卻屬不同型別；同樣的，DOH86d082(分離自痰液) 雖與DOH86d090同一天採檢，也屬不同型別。這或許反映出採檢部位的差異會造成型別的不同。長期採集自三名HIV病人的菌株(病人#1:CDC-F002000393和CDC- F002000397; 病人#2:CDC-F002000402, CDC-F002000404和CDC-F002000406; 病人

#3:CDC-F002000416和CDC- F002000419)，則全部分屬不同型別。顯示在採集間隔夠長時(1~3年)，念珠菌足以累積出足夠的變異性，以致於來自同一HIV病人的菌株均分屬不同型別。以MLST區分這些來自相同病人的菌株，雖然其最終的DST型別均不同，但細究其各基因的基因型仍可見親疏的不同。以N4醫院來自相同病人的菌株而言，以PFGE-*Bss*HII分型同型的MLST僅有2~3個基因型差異(DOH86f007及DOH86f044和DOH86d090及DOH86f022)；不同型別的則有5個基因型差異(DOH86f007及DOH86e040和DOH86d090及DOH86d082)。此外，CDC-F002000393 (HIV病人#18)與CDC- F002000393(病人#4)二株菌株用MLST及PFGE-*Bss*HII二種方法分型的結果相同，是屬巧合或具流病上意義，值得進一步探討。

c. 發展新型AFLP分子分型方法：

AFLP結果顯示，35株菌株僅能分出28種型別，其分型效果僅比PFGE-EK稍好，較諸PFGE-*Sfi*I、PFGE-*Bss*HII及rep-PCR分型效果均差(表五) (圖八)。且AFLP型別與院

內感染與否、分離部位、地理區來源, 或對fluconazole的抗藥性等無關，和個別病人亦無名現相關性。

(三)、探討感染HIV病患*C. albicans*之分子流行病學。

a. 應用PFGE-*Sfi*I及-EK分型方法：

我們利用 PFGE-*Sfi*I 及-EK 二種分型法分析來自 3 位 HIV 患者的 21 株 *C. albicans* 分離株(表九)。病人#1 於 1999, 2001, 和 2002 分別各分離二株菌株，所有這 6 株菌株均對 fluconazole 具感受性。病人#2 於 1999 和 2001 年各分別分離 3 株，2002 年分離出 4 株，這 4 株中 3 株(CDC-F003000400, CDC-F003000401 和 CDC-F003000402) 來自同一採檢棉棒，另一株則來自另一採檢棒。病人#2 分別在 1999 和 2002 年分離的菌株具較高的抗藥性(MICs from 16-256 μg/ml)至於 2001 年的分離株則抗藥性較低(0.125-1 μg/ml)。病人#3 於 2001 年分離 3 株，2002 年 2 株。除病人#3 於 2001 年的分離株有二株具高抗藥性 (MICs 32 和 128 μg/ml)外，其他分離株的抗藥性則較低(0.125 to 0.5 μg/ml)。病人 CD4 淋巴球數目從 15 至 448 不等。CD4 淋巴球數目最低的是病人#2 在 1999 年(CD4=15)及病人#3 在 2001 年(CD4=37)。在這些時間這二位病人也分別發展出念珠菌病，並接受抗

真菌藥物治療。

21 株菌株的核型圖譜詳見圖九。源自不同病人的菌株具有不同核型，而源自同一病人同一年度的菌株除 R 染色體外核型相同。R 染色體的差異是由於 rDNA repeats 的不同。即使來自同一採檢棉棒的不同菌株 R 染色體大小也有差異。同一病人在不同年分離的菌株核型則不同。例如, 病人#1 於 1999 年(lanes 5 和 6) 的菌株與 2002 年(lanes 1 和 2)和 2001 年(lanes 3 和 4) 的菌株的染色體 3 和 4 處有差異。病人#1 於 2002 年(lanes 1 和 2)和於 1999 年(lanes 5 和 6)的菌株 7 號染色體處有差異。病人#2 於 2002 年(lanes 7-10)和 1999 年(lanes 14-15)的菌株的 6 號和 7 號染色體處有差異。

利用 PFGE-*Sfi*I 分型方法可將 21 株臨床株區分為 3 大群。從病人#1 和#2 的結果顯示，即使分離間隔時間長達 3 年，菌株間仍分別有 85.5% 及 86.9%之相似類緣性(圖十)。2001 年從病人#3 分離之三株菌株(CDC-F003000417, CDC-F003000418, 和 CDC-F003000419)雖然對 fluconazole 的 MIC 值差異甚大，分別為 0.125, 128, 和 32 μg/ml，但這三株的 DNA 圖譜並無不同。

病人#3 上的 5 株菌株中，三株分離自 2001 年病人有嚴重念珠菌症，二株分離自 2002 年(CDC-F003000420 和 CDC-F003000416)，當時病人無明顯念珠菌症。然而這 5 株菌株仍有 79.6% 的類緣相關性，顯示它們為相關株系。同樣的情形也可在病人#2 上觀察到。同一病人的念珠菌致病及移生株間的類緣性仍高達 86.9% 。

b. 應用rep-PCR分型方法：

以 rep-PCR 探討 45 株來自 HIV 病人念珠菌的型別特性並以 46 株來自非 HIV 病人的菌株做對照，所有菌株皆是來自不同病人(表九)。結果顯示來自 HIV 病人的 *C. albicans* 菌株皆具有較簡單的指紋圖譜，且共可分為 A(24 型, 53.3%), F(9 型, 20%), E(8 型, 17.8%), C(4 型, 8.9%)四種分子型;來自非 HIV 病人的菌株則有較多片段，且共可分為 C(24 型, 52.2%), G(11 型, 23.9%), D(4 型, 8.7%), H(3 型, 6.5%), F(2 型, 4.3%), E(1 型, 2.2%)和 B(1 型, 2.2%)七種分子型。且以樹狀圖分析時，除少數菌株外(屬於 C,E, F 型別的)，來自 HIV 病人與非 HIV 病人的菌株會各別集群(clustering) (圖十一)。

四、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。我們與國衛院合作收集全省代表性菌株，精確鑑定種別並測定這些菌株的抗藥性台灣地區病原真菌之種別及抗藥性分佈方面。

我們也利用真菌核醣體DNA基因或間隔轉錄區ITS序列，發展有助於臨床醫生投藥的PCR-EIA快速鑑定方法方面，以臨床分離菌株及血瓶培養檢體測試結果，所設計三組抗藥性引子具有高專一性，可將菌株依其對fluconazole不同抗藥性加以區分，靈敏度可達10fg/100μl以下，鑑定結果與綜合germ tube , Viteck及API20C的生理生化鑑定法結果符合度達100％。此外，混合感染的情形也可被清楚地鑑定出來，這對涉及抗藥性不同菌株的混合感染尤其重要。此 PCR-EIA方法可謂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，使得檢驗實驗室之工作流程更形精簡流暢，更有助於臨床投藥防治之

參考。

評估新型 LAMP (Loop-mediated Isothermal Amplification) 快速鑑定法方面，收集整理文獻資料，並初步評估 LAMP 快速鑑定法之實用性。依據其他 LAMP 文獻的電泳圖顯示，成功的 LAMP 反應 DNA 在電泳後應該呈現多層次片段，且從 well 開始就有「拖曳」現象，但是自行設計的 primer 及製作的反應結果，相形之下效能顯的極差。雖然電泳結果也有「拖曳」的情況，但 DNA 的產能不佳，只能說 primer 有用，功效卻無法肯定。

另外以相同的實驗方法對真菌 Candida 測試 LAMP 的可行性，發現效能比較差，且檢驗的結果不穩定，在引子的設計上，26S rDNA 序列比對後，發現不論種間或是種內的比對結果較雜亂，又加以引子設計條件嚴苛，引子使用的理想性較差，還是要多設計引子做測試，以篩選出最適合作為檢驗用的引子。在重複 LAMP 文獻的實驗後，發現日本廠商的試劑與實驗室建立的試劑效能有極大的差別，因此在引子測試上，結果與效能仍有待要引進日本廠商的試劑套組再做測試以確定。

開發分型技術，有助於瞭解病原間之類緣性及分子流行病學，進而釐清病原之傳播途徑。近年來，病原菌隨著國際旅行、候鳥的遷徙或全球食物供應鏈傳播的案例日益增加，如SARS，禽流感，MRSA, 和食因性病原如*E. coli*與*C. jejuni* [103]等，益加彰顯了建立適當分型技術於國際疫情監測及防治上之重要性。

本論文共計開發PFGE-EK、PFGE-*Sfi*I、PFGE-*Bss*HII、rep-PCR、MLST及AFLP等六種分子分型方法。除比較各分型方法之鑑別力及適用性外，本研究也是首度嘗試瞭解國內白色念珠菌型別分佈及查明是否不同特性(例如抗藥性、地源性、分離來源、是否院內感染)可歸類為某種台灣特定的型別。

分型鑑別力比較顯示，最有識別力的是MLST，接著是PFGE-*Bss*HII與PFGE-*Sfi*I，rep-PCR方法居次，AFLP居其後，而PFGE-EK識別能力最差。

MLST分型方法MLST分型方法的原理是基於*Candida*的持家基因會由於突變或重組產生變異，綜合多個持家基因的變異，可據此決定菌株間之類緣性。每株菌

株依其ST相互比較，可據此瞭解菌株間核苷酸置換改變的情形[104,105]。MLST的資料明確並利於儲存在一個中心資料庫，可很方便地經由網路交換分子型別資訊，有利於全球流病監測資料之建立。如在M RSA上MLST已被用於追蹤病原獲致抗藥性和跨洲傳播演化的歷程 [106,107]。病原真菌方面除*C. albicans* 外，許多種的MLST已被發展出來，例如*C. glabrata* [108], *Histoplasma capsulatum*, *Aspergillus flavus*, *Coccidoides immitis* [109]和*Fusarium oxysporum* 菌群[110]。

PFGE酶切分型法略遜於MLST，且若落實實驗室間的比對，需有嚴格的標準化程序，以保證比對的品質及再現性。本研究使用的三個PFGE為本的方法有高一致性，且均能鑑別出來自同一病人的類緣相關菌株。短期內來自同一病人的菌株屬於同一獨特之型別。如1~2年內來自3個HIV病人的7株菌株依PFGE-*Bss*HII 分型方法可歸為3 種型別。藉由PFGE分型法我們也清楚地顯示每個菌株的DNA型別與個別病人有關而和院內感染與否, 分離部位, 地理區來源, 或對fluconazole的抗藥性等無關這也與先前的報告一致[111]。

PFGE相關方法主要在於在區別DNA的指紋片段，主要原理乃基於個別酶切多型、易位[112]，或不包含rDNA染色體的重組或rDNA作用子(cistron)非互補的重組[113] 的結果。限制酶酵素的選擇也甚為重要，不同菌株或菌種適用的限制酶不一樣。理想的指紋法片段在廣泛分子範圍內應包含20-30條。而限制酶*Not*I，或者 *Sma*I [114]在窄的分子範圍內大約有10條片段，並不合適。PFGE-*Sfi*I 與PFGE-*Bss*HII各辨認13和6 bp的限制酶切位，提供感染性*C. albicans*分子分型更多分析能力。限制酶*Sfi*I產生18-22條清晰片段(平均20條)，可清楚的區別片段範圍由40到1100kb。PFGE-*Bss*HII片段更多(平均31條，範圍由50到1000kb)，區別能力最強，但相對要比PFGE-*Sfi*I花更多時間去辨認及計數其片段。另一種替代方案可為先使用*Sfi*I當作第一分型酵素，無法區分的區別的再以*Bss*HII分型，若結合PFGE-*Sfi*I與PFGE-*Bss*HII兩方法識別能力可達1.0。

PFGE-EK為識別能力最差的方法，扣除R染色體外，在53株中僅區分出19型，如果把R染色體列入，可分出25型。然而，我們觀察到R 染色體長度變化頻率甚高，其中R染色體的大小依rDNA的複本數多寡約2.5至4 Mbp不

等[115]，而rDNA的複本數(copy number)會隨每次細胞分裂而變化。

過去真菌文獻中，尚未有系統性的比較rep-PCR與PFGE分型法。rep-PCR分型方法，與RAPD方法均屬於PCR分型法，甚為快速簡便，適合例行性高通量使用。不過，RAPD分型使用短且非特一性引子，於非嚴苛情況下進行PCR反應，有缺乏再現性的問題[116]。我們挑選rep-PCR主要是因為其分型方法基於不同的原理，主要是依據內重複序列長度多型分析(inter-repetitive sequence length polymorphism分析)，且再現性高。我們的資料顯示，用Ca-21與Ca-22引子對*Candida*基因反覆PCR增幅，會產生簡單片段且識別力比限制酶切的PFGE低。rep-PCR與三種PFGE基本法一致性低。然而，當rep-PCR被使用當作第二個分型法，補充EK, PFGE-*Sfi*I 與PFGE-*Bss*HII三個基本分型法時，分別可獲得高識別力指數0.996、0.998和0.998。此結果顯示出若結合二種作用原理不同的分型方法(如PFGE- EK+rep-PCR或PFGE-*Sfi*I+rep-PCR)亦可提高鑑別力。

發展新型AFLP分子分型方法，其鑑別力僅略勝

rep-PCR。由於技術剛建立，數據較少有關其適用性及再現性仍有待更多實驗來確認。AFLP之進一步發展為藉由AFLP或c-DNA-AFLP技術鑑別出負責特別表現型的基因體或轉錄質體特異性片段。

綜言之，我們的結果顯示每個分離株的DNA分型是病人特有的，而非像引起院內感染能力或血流感染的特別基因型占優勢。此研究收集來自於台灣四處的18間醫院，因此有不同地域性的代表分離株，不同醫院基因型廣泛分散，說明在基因型與醫院/地域性兩者間並無相關性。瞭解抗藥性菌株基因型的變化可能有助於抗真菌的治療療法，然而我們目前的結果顯示，型別與抗藥性之間並無相關性。著個發現與許多報告一致的[117]。可能*C. albicans*對fluconazole藥物敏感性的變化是由於染色體DNA相關抗藥性基因的微小變化有關[118]，用我們的基因指紋分型方法區分開來。若想偵測突變、缺失或者插入是否與抗藥性有關，基因定序分析應該比基因指紋法更適合[119]。

C. albicans 在 HIV 患者的感染十分重要。然其分子流行病學仍未全然闡明。應用 PFGE-EK 及 PFGE-*Sfi*I 分型法建立從三位 HIV 患者分離之 21 株 *C. albicans* 菌株間之遺傳類

緣關係，長期追蹤(超過 3 年)愛滋病人之白色念珠菌型別變化，發現每一位愛滋病患身上所帶菌株均屬其專一的型別，其間或有接受藥物治療，抗藥性亦有起落，念珠菌症(candidiasis)亦間歇發作，但同一病人的型別仍變化甚小。念珠菌症之發作與否與菌的型別變化相關性不大，而與病患的 CD4 指數低下與否息息相關。念珠菌症曾見在愛滋病患伴侶間平行傳播感染之報導，利用本論文所建立之分型方法，可為監測念珠菌在愛滋高危險群間傳播之有效工具。

對 fluconazole 感受株和抗藥株間及侵襲感染株和移生株間屬同類緣株系。一種可能解釋是源自菌株染色體細微的改變染色體，反之亦然。而這種細微的改變無法反映在我們的分子分型方法。

雖然，曾見報導同一病人 *C. albicans* 菌株的核型(karyotypic patterns)即使間隔長達 140 天仍維持不變，然而，我們的研究卻顯示並非所有源自同一病人的菌株核型均維持一致。不一致處包括 R 染色體的高頻率長度變化及染色體常發生的位移(translocation)有關[120] (3, 4, 6, 和 7 號染色體)。染色體的高頻率變化與藥物暴露的久暫有相關。培養於含 fluconazole 的培養基之下，可能發生 4 號染

色體失去一同源片段和 3 號染色體增加片段。*C. albicans* 的抗藥性是由染色體遺傳主導。一般已知可能機制有三: 1 降低藥物的累積，這包括阻止藥物運入細胞及活化藥物運出細胞的功能； 2 改變藥物的作用標的，這包括促使藥物標的基因突變、標的基因的大量表現及, 和藉由改變同一酶路徑的其他酶來規避藥物標的酵素 [121,122]。我們現有的數據顯示病人同一年分離的菌株，即使對 fluconazole 的抗藥性高低不同，其 DNA 型別仍為同類緣株系，故抗藥性與 DNA 型別無關。

雖然曾見抗藥性 *C. albicans* 菌株平行傳播的報導，我們的研究顯示，在 HIV 病人長期移生的 *C. albicans* 菌株應為內源性感染之可能居多 [123]。要能成功感染增加病原性，伺機性病原包括 *C. albicans* 需能躲避寄主內免疫系統的攻擊、存活並繁衍及散佈，包括要能提升他們的黏附性並分泌水解酵素。然而，在宿主免疫缺損時，生物如何從共生菌變病原菌，反之，宿主免疫恢復時，生物如何從病原菌變無害的移生菌，這些均值得進一步探討。

雖然 PFGE 方法的結果常常取決於實驗室，然而隨著標準化擬定，實驗室間的比對仍然可行，且對流行爆發研究是

有用的且具成本效益的工具，並可回答長期的流病問題。美國 CDC 建立以 PFGE 技術的 PulseNet 於食物中毒流病調查上之應用情形，示範了 PFGE 技術經標準化後，作為食因性病原追溯感染源頭及發揮預警效果，避免疫情擴大之實例探討。以序列原理分型方法如 MLST 的缺點為成本高，但未來隨著更低成本更高通量 DNA 定序技術之出現，將可獲得全球的一致性而且普遍化，利於普及。本文的資料也是台灣甚至亞洲首度有關 *C. albicans* 的 MLST 資料。MLST 優點為資訊明確，比對簡易，但仍有價昂、鑑別力不高及需事先已知病原序列之缺點，阻礙其普及化。MLST 著重在少數基因(通常 6-8 條)的序列分析，資訊少但精確。與單核苷酸多型性(single nucleotide polymorphism, SNP)比較下，MLST 的優點為可較全面且正確地瞭解多型性位點的情形和可偵測出新的多型性位點。至於 Array 則涉及數千條基因之雜和每條代表一個 ORF，較為廣泛，但僅能知道 match/mismatch。晚近則有結合二者的 MLST 陣列 MLST-like array 之發展可進一步擴大化分型的通量 [124-126]。

五、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 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，應可提供臨床投藥判斷的輔助。

為了鑑定病原菌的族群關係，選擇適當的分子分型方法是必須的。本研究獲得的資料為可幫助建立將來台灣監測的基準，也可當作其他分型方法比較的一個依據。所發展之高鑑別力分型法經實際檢體驗證，確能區分出流病相關菌株，這將可成為流病調查監測的可靠工具。且可為未來新分型方法之比較基礎。所建立之全國性分型資料，可成為國內、國際型別交流比較之參考。本研究成果顯示，MLST 鑑別效果最好且已有國際性網站供型別資料上傳及擷取，利於全球性比對及流病資料之交流。不過完成一株分型約需新台幣

5~6000 元仍屬昂貴，未來欲調查念珠菌之群聚感染，建議可先將收集菌株進行 PFGE 限制酵素方法分析，找出的主要或指標性菌株型別再進行 MLST 分析，並上傳做國內及國際性比對。

監測念珠菌在 HIV 患者身上流行感染情形除了有助於防治念珠菌改善患者生活品質外，我們初步的 rep-PCR 研究也顯示藉由分析念珠菌的型別(簡單的指紋片段)或許可有助於發掘潛在的 HIV 患者。這部分假說的確認有待進一步的加強研究。

病原真菌基因序列之相繼解碼，提供了分子診斷及分型技術莫大的契機。經由序列的搜尋比對，可依臨床檢驗或投藥需要設計出鑑別不同種之病原真菌的專一性引子及探針。此外進一步研發 AFLP 鑑別基因體及轉錄質體特異性片段，應也能有助於提昇監測及防治效率。

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七、 圖、表

表一、PCR 和 PCR-EIA 試驗使用的引子及探針序列

引子或探針	序列（5′ 至 3′）	GenBank 編號	核苷酸標幟
引子			
ITS1	TCC GTA GGT GAA CCT GCG G		5.8S rDNA universal forward primer
ITS4	TCC TCC GCT TAT TGA TAT GC		28S rDNA universal reverse primer
探針			
ITS3- B	GCA TCG ATG AAG AAC GCA GC		5′-Biotin-labeled universal capture probe
CFS	GGG TTT GGT GTT GAG CAA TAC G	AY196001, AF287909, AF321539, AJ249484	5′-end-labeled digoxigenin probe for *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. dubliniensis*
CFM	TCG GTG TTG ATC TAG GAA GGG A	AF167993	5′-end-labeled digoxigenin probe for *C. glabrata*
CFR	AGC GAG TGT TGC GAG ACA ACA A	AF246989	5′-end-labeled digoxigenin probe for *C. krusei*

表二、以PCR-EIA 分析DNA 探針的種別專一性

Genomic DNA tested	Mean A_{650} for EIA diction of PCR products with probe		
	CFS	CFM	CFR
C. albicans	**1.026**	0.070	0.058
C. dubliniensis	**1.724**	0.067	0.059
C. parapsilosis	**1.639**	0.066	0.056
C. tropicalis	**1.040**	0.069	0.056
C. glabrata	0.068	**1.712**	0.058
C. krusei	0.057	0.068	**1.281**

[a] 取5ng的純化DNA (見材料與方法)進行PCR增幅反應。

[b] 每種特異性探針的陽性閥值(cut-off，此處為0.077)是取陰性對照平均值加二倍標準偏差。粗體數字代表DNA探針與PCR產物有雜合反應。

表三、各臨床菌株的 A_{650} 值

種別 [a]（測試菌株數）	Mean A_{650} after reaction with probe[b]		
	CFS	CFM	CFR
C. albicans (6)	**1.043**	0.069	0.057
C. dubliniensis (1)	**1.170**	0.072	0.063
C. parapsilosis (5)	**1.506**	0.066	0.059
C. tropicalis (6)	**0.787**	0.066	0.060
C. glabrata (5)	0.065	**1.319**	0.058
C. krusei (4)	0.064	0.067	**1.097**
C. parapsilosis＋*C. glabrata* (1)	**0.475**	**1.388**	0.067
C. glabrata＋C. albicans (2)	**0.174**	**1.477**	0.075
C. glabrata＋*C. krusei* (1)	0.075	**0.892**	**0.564**

[a] 取5ng的純化DNA (見材料與方法)進行PCR增幅反應。

[b] 每種特異性探針的陽性閥值（cut-off，此處為0.111）是取陰性對照平均值加二倍標準偏差。粗體數字代表探針與PCR產物有雜合反應。

表四、比較Candida spp.用BacT/Alert血瓶培養等傳統表現型及用PCR-EIA方法之鑑定結果

傳統表現型鑑定	血瓶檢體數	Specific-probe identification		
		CFS（＋）	CFM（＋）	CFR（＋）
C. albicans	13	13	0	0
C. parapsilosis	6	6	0	0
C. tropicalis	6	6	0	0
C. glabrata	8	1	7	0
C. krusei	9	0	0	9
C. tropicalis＋*C. krusei*	1	1	0	1

表五、DNA types of 53 isolates of *C. albicans*.

Code No.	PFGE karyotype	PFGE-SfiI	PFGE-BssHII	Rep-PCR	AFLP	Source of isolation	region* /hospital	Fluconazol MIC(μg/ml)	Nosocomial	HIV status
CDC F003000062	A1	B1	C1	D1	E1	Urine	N3	0.125	Yes	No
CDC F003000064	A1	B2	C2	D1	E2	Urine	N3	0.125	Yes	No
CDC F003000088	A2	B3	C3	D2	E3	Sputum	M3	0.125	No	No
CDC F003000112	A3	B4	C4	D3	E1	Sputum	S3	0.125	No	No
CDC F003000113	A3	B5	C5	D4	E4	Sputum	S3	0.125	No	No
CDC F003000114	A3	B5	C6	D5	E5	Urine	S3	0.125	No	No
CDC F003000139	A4	B6	C7	D6	E6	Urine	S4	64	Yes	No
CDC F003000142	A5	B7	C8	D7	E2	Wound	S4	0.25	No	No
CDC F003000147	A6	B8	C9	D8	E7	Sputum	M2	0.25	No	No
CDC F003000148	A7	B9	C10	D2	E4	Stool	M2	ND	No	No
CDC F003000149	A8	B10	C11	D9	E2	Urine	M2	0.25	Yes	No
CDC F003000150	A9	B11	C12	D10	E8	Sputum	M2	0.25	Yes	No
CDC F003000174	A3	B12	C13	D2	E9	Urine	N7	0.25	No	No
CDC F003000184	A10	B13	C14	D11	E10	Pleural effusion	S2	0.25	Yes	No
CDC F003000186	A11	B14	C15	D10	E11	Sputum	S2	0.5	No	No
CDC F003000204	A12	B15	C16	D12	E12	Sputum	S1	0.125	No	No
CDC F003000219	A13	B16	C17	D12	E11	CVP tip	N8	0.125	0.25	No
CDC F003000220	A8	B10	C18	D12	E4	Urine	N8	0. 5	Yes	No
CDC F003000225	A8	B10	C19	D13	E13	Urine	N8	0.25	Yes	No
CDC F003000232	A3	B12	C20	D13	E14	Urine	N2	0.125	No	No
CDC F003000233	A3	B17	C21	D14	E15	Urine	N2	0.125	Yes	No
CDC F003000234	A14	B18	C22	D15	E11	Urine	N2	0.25	Yes	No
CDC F003000235	A4	B6	C23	D16	E16	Wound	N2	0.25	No	No
CDC F003000242	A9	B19	C24	D15	E17	Wound	N1	64	No	No
CDC F003000243	A8	B20	C11	D17	E18	Urine	N1	1	No	No

CDC F003000244	A8	B10	C25	D17	E2	Ascites	N1	0.125	No	No
CDC F003000268	A8	B21	C26	D18	E11	Sputum	N6	32	No	No
CDC F003000269	A15	B22	C27	D19	E11	CVP	N6	0.125	Yes	No
CDC F003000270	A7	B23	C28	D19	E18	Sputum	N6	0.125	No	No
CDC F003000293	A4	B24	C29	D20	E12	Sputum	N5	0.125	No	No
CDC F003000294	A16	B25	C30	D21	E19	Urine	N5	0.25	No	No
CDC F003000297	A8	B10	C31	D16	E20	Urine	M1	0.25	No	No
CDC F003000301	A17	B26	C32	D18	E21	CVP	M1	4	Yes	No
CDC F003000311	A4	B27	C22	D22	E22	Wound	N9	0.5	No	No
CDC F003000313	A18	B28	C33	D23	E23	Urine	N9	0.125	No	No
CDC F003000314	A18	B29	C34	D24	E19	Sputum	N9	0.25	No	No
CDC F003000352	A17	B30	C35	D25	E24	Blood	E1	0.25	No	No
CDC F003000353	A17	B31	C35	D25	E25	Urine	E1	0.125	No	No
CDC F003000354	A17	B31	C36	D25	E26	Urine	E1	0.125	No	No
CDC F003000379	A6	B8	C37	D26	E8	Sputum	E2	0.25	Yes	No
CDC F003000380	A17	B32	C38	D27	E!3	Sputum	E2	0.25	Yes	No
CDC F003000381	A6	B33	C37	D28	E11	Sputum	E2	0.25	Yes	No
CDC F003000393	A4	B6	C7	D29	E27	Oral	N4	0.25	No	Yes
CDC F003000394	A4	B6	C7	D29	E25	Oral	N4	0.125	No	Yes
CDC F003000395	A4	B6	C7	D29	E24	Oral	N4	0.125	No	Yes
CDC F003000396	A4	B6	C7	D29	E23	Oral	N4	0.5	No	Yes
CDC F003000399	A8	B34	C39	D30	E2	Oral	N4	64	No	Yes
CDC F003000400	A8	B34	C39	D30	E24	Oral	N4	16	No	Yes
CDC F003000401	A8	B34	C39	D30	E2	Oral	N4	16	No	Yes
CDC F003000402	A8	B34	C39	D30	E2	Oral	N4	256	No	Yes
CDC F003000418	A19	B35	C40	D31	E20	Oral	N4	0.125	No	Yes
CDC F003000419	A19	B35	C40	D31	E13	Oral	N4	128	No	Yes
CDC F003000420	A19	B35	C40	D31	E28	Oral	N4	32	No	Yes

* N: north, S: south, M: middle, E: east

表六、Oligonucleotide primers for amplification 和 sequencing of loci used in *C. albicans* MLST scheme

Locus	Primer	Sequence 5'→3'	片段大小 (bp)	基因型數	核甘酸變異數(%)
CaACC1	466 forward	GCAAGAGAAATTTTAATTCAATG	407	9	5
	466 reverse	TTCATCAACATCATCCAAGTG			
CaVPS13	552 forward	TCGTTGAGAGATATTCGACTT	403	18	14
	552 reverse	ACGGATGGATCTCCAGTCC			
CaGLN4	598 forward	GAGATAGTCAAGAATAAAAAAGT	404	14	8
	598 reverse	ATCTCTTTCATCTTTTGGACC			
CaADP1	904 forward	GAGCCAAGTATGAATGATTTG	443	11	14
	904 reverse	TTGATCAACAAACCCGATAAT			
CaRPN2	1041 forward	TTCATGCATGCTGGTACTAC	306	13	12
	1041 reverse	TAATCCCATACCCAAAGCAG			
CaSYA1	1369 forward	AGAAGAATTGTTGCTGTTACTG	391	19	11
	1369 reverse	GTTACCTTTACCACCAGCTTT			

表七、菌株特性和 MLST 結果

病人編號	菌株編號	來源		Fluconazole	PFGE		Allele no.						
		部位	醫院	MIC(μg/ml)	-BssHII	HIV	*CaACC1*	*CaVPS13*	*CaGLN4*	*CaADP1*	*CaRPN2*	*CaSYA1*	DST
1	CDC-F003000064	Urine	N3	0.25	1	否	1	10	9	1	6	1	1
2	CDC-F003000089	Sputum	M3	0.25	2	否	1	2	4	5	5	5	2
3	CDC-F003000112	Sputum	S3	0.125	3	否	1	7	8	10	9	8	3
4	CDC-F003000139	Urine	S4	64	4	否	1	11	5	2	6	3	4
5	CDC-F003000142	Wound	S4	0.25	5	否	1	7	3	10	10	8	5
6	CDC-F003000184	Pleural effusion	S2	0.25	6	否	4	9	11	7	12	2	6
7	CDC-F003000219	CVP tip	N8	0.25	7	否	4	8	7	6	3	13	7
8	CDC-F003000242	Wound	N1	64	8	否	5	12	10	11	11	16	8
9	CDC-F003000268	Sputum	N6	32	9	否	2	3	4	3	5	15	9
10	CDC-F003000297	Urine	M1	0.25	10	否	1	11	6	8	6	4	10
11	CDC-F003000298	Pental swab	M1	0.25	11	否	1	10	5	1	6	1	11
12	CDC-F003000314	Sputum	N9	0.25	12	否	1	11	5	2	6	4	12
13	CDC-F003000352	Blood	E1	0.25	13	否	1	5	3	4	5	10	13
14	CDC-F003000354	Urine	E1	0.125	14	否	1	1	1	10	10	12	14
15	CDC-F003000379	Sputum	E2	0.25	15	否	4	4	1	1	4	14	15
16	DOH86e040	Rectal swab	N4		22	否	7	10	5	1	6	1	22
16	DOH86f007	Sputum	N4		23	否	8	15	1	1	14	18	23
16	DOH86f044	Pleural effusion	N4		23	否	4	16	3	1	14	18	24
17	DOH86d082	Sputum	N4		24	否	6	13	12	10	13	12	25
17	DOH86d090	Rectal swab	N4		25	否	1	14	3	10	8	17	26
17	DOH86f022	Anal swab	N4		25	否	1	7	3	10	9	17	27
18	CDC-F002000393	Oral	N4	0.25	4	是	1	11	5	2	6	3	4
18	CDC-F002000397	Oral	N4	0.125	16	是	3	8	2	2	7	11	16
19	CDC-F002000402	Oral	N4	256	17	是	1	6	3	1	8	6	17
19	CDC-F002000404	Oral	N4	0.125	18	是	1	6	3	10	8	8	18
19	CDC-F002000406	Oral	N4	64	19	是	1	6	3	9	8	9	19
20	CDC-F002000416	Oral	N4	0.25	20	是	1	1	3	9	1	7	20
20	CDC-F002000419	Oral	N4	128	21	是	1	1	1	9	1	8	21
	ATCC10053				26		9	17	13	1	3	19	28
	ATCC90028				27		1	18	14	10	13	12	29

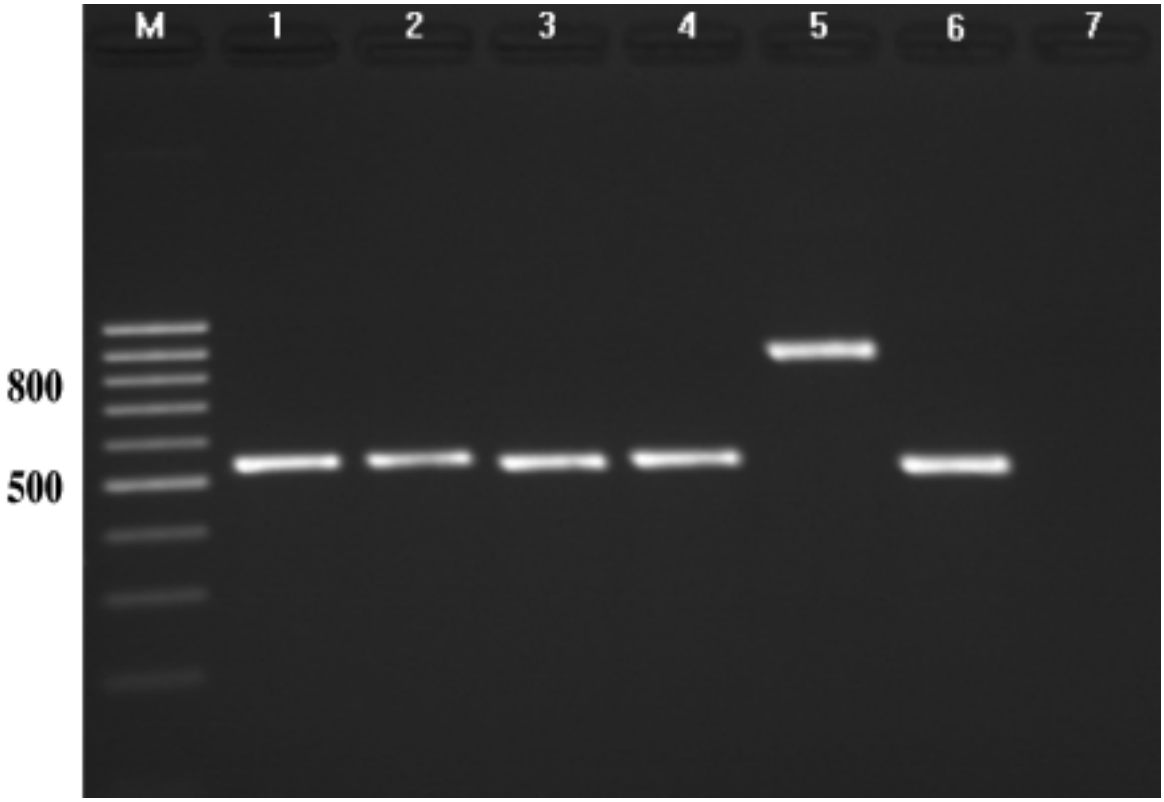
表八、從 3 位 HIV 病人分離的 21 株 *Candida albicans* 臨床菌株之特性

病人	年齡	菌株	PFGE	MIC (μg/ml)	年代	CD4 數目	抗反轉錄病毒治療	念珠菌症	抗真菌治療
1	37	CDC-F003000397	A-1	0.125	1999	223	Yes	No	No
1	37	CDC-F003000398	A-1	0.125	1999	223	Yes	No	No
1	39	CDC-F003000395	A-2	0.125	2001	448	Yes	No	No
1	39	CDC-F003000396	A-2	0.5	2001	448	Yes	No	No
1	40	CDC-F003000393	A-2	0.25	2002	ND	Yes	No	No
1	40	CDC-F003000394	A-2	0.125	2002	ND	Yes	No	No
2	29	CDC-F003000406	B-1	64	1999	15	No	Yes	Fluconazole
2	29	CDC-F003000407	B-1	64	1999	15	No	Yes	Fluconazole
2	29	CDC-F003000408	B-1	16	1999	15	No	Yes	Fluconazole
2	31	CDC-F003000403	B-3	0.5	2001	364	Yes	No	No
2	31	CDC-F003000404	B-3	0.125	2001	364	Yes	No	No
2	31	CDC-F003000405	B-3	1	2001	364	Yes	No	No
2	32	CDC-F003000399	B-2	64	2002	ND	No	No	No
2	32	CDC-F003000400	B-2	16	2002	ND	No	No	No
2	32	CDC-F003000401	B-2	16	2002	ND	No	No	No
2	32	CDC-F003000402	B-2	256	2002	ND	No	No	No
3	45	CDC-F003000418	C-2	0.125	2001	37	Yes	Yes	Mycostatu
3	45	CDC-F003000419	C-2	128	2001	37	Yes	Yes	Mycostatu
3	45	CDC-F003000420	C-2	32	2001	37	Yes	Yes	Mycostatu
3	46	CDC-F003000416	C-1	0.25	2002	270	Yes	No	No
3	46	CDC-F003000417	C-1	0.5	2002	270	Yes	No	No

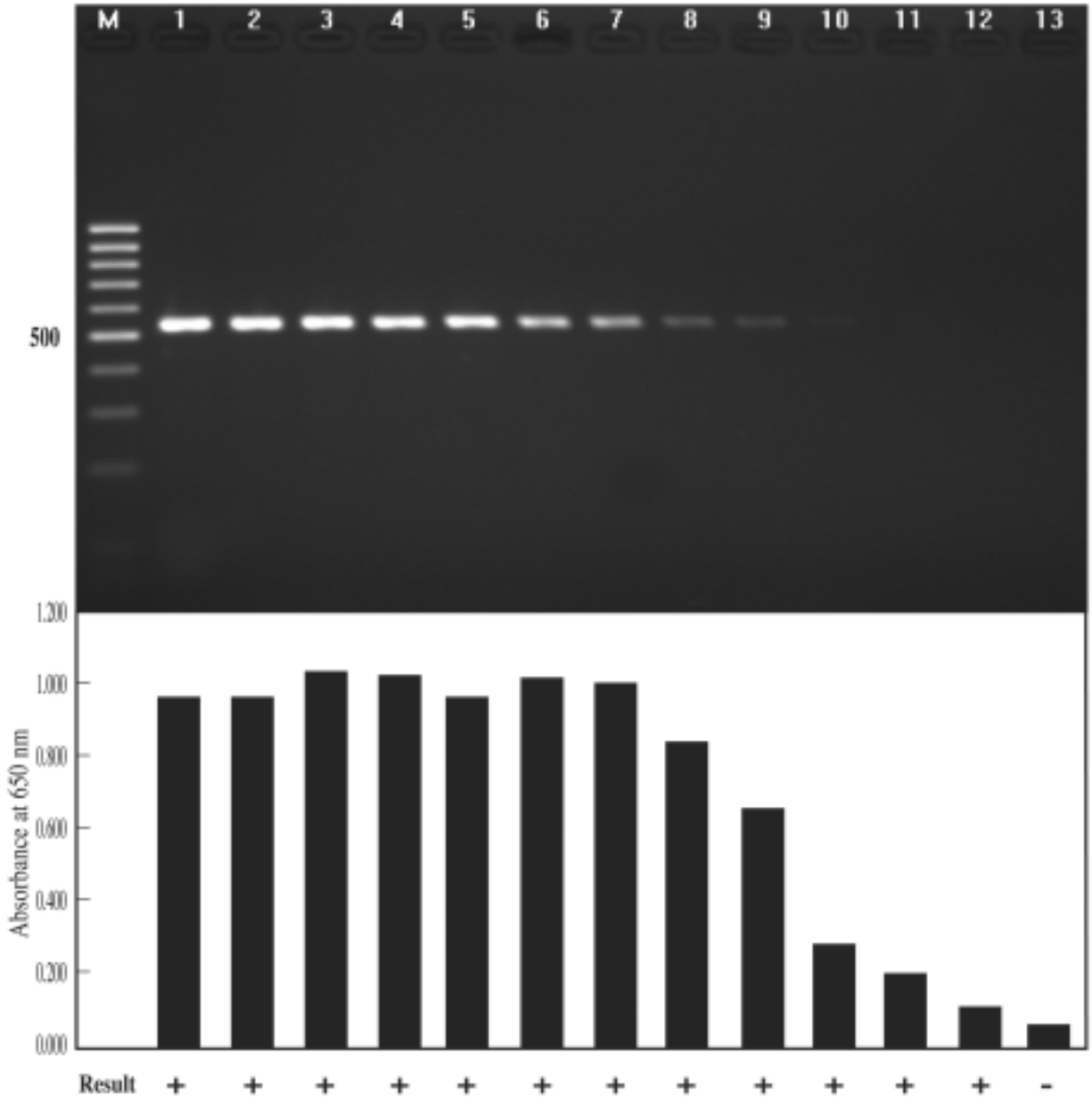
表九、以 rep-PCR 分析 45 株來自 HIV 病人及 46 株來自非 HIV 病人念珠菌的特性。

Code no.	Fluconazole(MIC)	Amphotericin B (MIC)	HIV status	Sab=0.8 cluster
CDC-F003000062	0.125	0.5	–	G
CDC-F003000063	0.5	0.5	–	G
CDC-F003000064	0.125	1	–	G
CDC-F003000065	0.25	1	–	G
CDC-F003000066	0.25	0.5	–	G
CDC-F003000087	0.25	0.5	–	G
CDC-F003000088	0.125	1	–	G
CDC-F003000089	0.25	0.25	–	G
CDC-F003000090	0.25	0.25	–	G
CDC-F003000091	0.25	0.5	–	G
CDC-F003000092	0.125	1	–	C
CDC-F003000094	0.25	0.25	–	C
CDC-F003000099	0.125	0.5	–	C
CDC-F003000102	0.125	0.5	–	C
CDC-F003000103	8	0.5	–	B
CDC-F003000104	0.125	0.5	–	C
CDC-F003000105	0.25	0.5	–	C
CDC-F003000106	1	0.5	–	F
CDC-F003000108	8	0.5	–	D
CDC-F003000109	0.25	1	–	E
CDC-F003000110	8	0.5	–	D
CDC-F003000113	0.125	0.5	–	C
CDC-F003000114	0.125	0.5	–	C
CDC-F003000115	0.25	0.5	–	C
CDC-F003000116	0.125	0.5	–	C
CDC-F003000122	64	0.5	–	C
CDC-F003000137	1	0.5	–	C
CDC-F003000138	0.25	1	–	F
CDC-F003000139	64	0.5	–	D
CDC-F003000141	16	0.5	–	D
CDC-F003000142	0.25	0.5	–	C
CDC-F003000143	0.125	0.5	–	C
CDC-F003000147	0.25	0.5	–	C
CDC-F003000148	ND	ND	–	C
CDC-F003000149	0.25	0.5	–	G
CDC-F003000174	0.25	0.5	–	H
CDC-F003000175	0.125	0.5	–	H
CDC-F003000182	0.125	0.5	–	H
CDC-F003000183	0.25	0.5	–	C
CDC-F003000205	16	0.25	–	C
CDC-F003000218	0.125	0.25	–	C
CDC-F003000220	0.5	0.25	–	C
CDC-F003000232	0.125	0.25	–	C
CDC-F003000233	0.125	0.25	–	C

CDC-F003000242	64	0.25	−	C
CDC-F003000255	0.125	0.5	−	C
YH010034	0.25	0.5	+	A
YH010036	0.125	0.5	+	A
YH010040	0.125	1	+	A
YH010041	0.5	1	+	A
YH010042	0.25	1	+	A
YH010079	1	0.5	+	A
YH010081	0.125	0.5	+	A
YH010160	0.5	0.5	+	A
YH010162	0.25	0.5	+	A
YH010203	0.5	0.5	+	A
YH010214	0.125	0.5	+	A
YH010215	0.25	0.5	+	A
YH010217	0.125	0.5	+	F
YH010244	0.25	0.5	+	F
YH010253	0.25	0.5	+	F
YH010255	0.5	0.5	+	F
YH010286	0.125	0.5	+	C
YH010336	0.25	0.5	+	C
YH010337	0.125	0.5	+	C
YH010339	0.125	0.5	+	C
YH010371	0.125	0.5	+	E
YH020002	2	1	+	E
YH020004	0.25	0.5	+	E
YH020044	0.25	0.5	+	E
YH020045	0.25	0.5	+	E
YH020091	0.25	0.5	+	E
YH020092	0.5	0.5	+	E
YH020126	0.25	1	+	A
YH020265	0.5	0.5	+	E
YH020347	0.125	0.5	+	F
YH020348	0.25	1	+	F
YH020358	0.5	1	+	F
YH020374	0.5	1	+	F
YH020409	1	2	+	F
YH990004	0.25	0.5	+	A
YH990010	0.25	0.5	+	A
YH990011	0.125	0.5	+	A
YH990057	0.125	0.5	+	A
YH990127	0.25	0.5	+	A
YH990142	0.25	0.5	+	A
YH990144	0.125	0.5	+	A
YH990166	0.125	0.5	+	A
YH990168	0.25	1	+	A
YH990175	0.125	0.5	+	A
YH990176	0.125	0.5	+	A

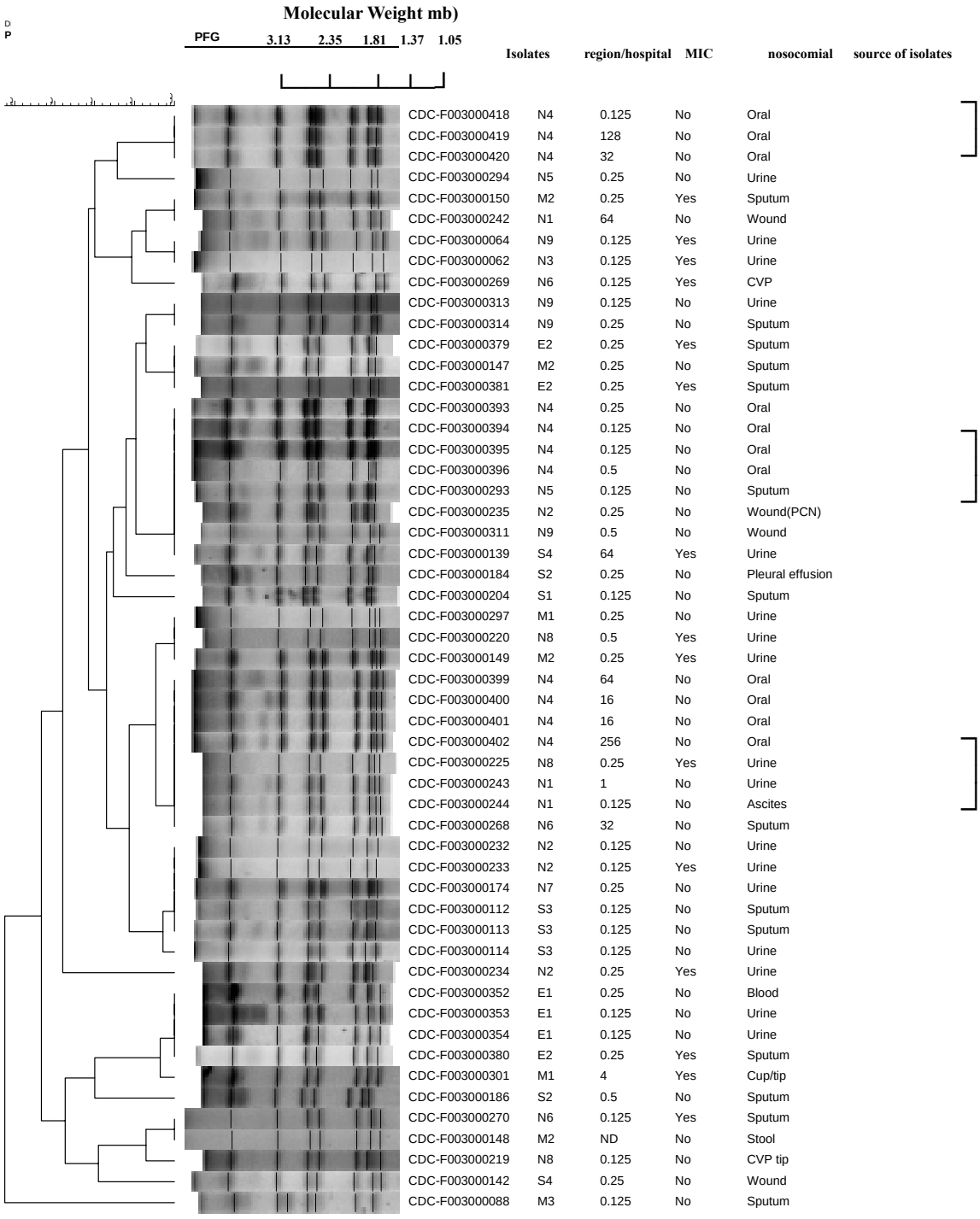


圖一、泛真菌ITS1和ITS4引子的增幅產物。Lanes: M, molecular size markers MassRuler^{TM} DNA Ladder; 1, *C. albicans*, 2, *C. parapsilosis*; 3, *C. tropicalis*; 4, *C. dubliniensis*, 5, *C. glabrata*; 6, *C. krusei*; 7, negative control.

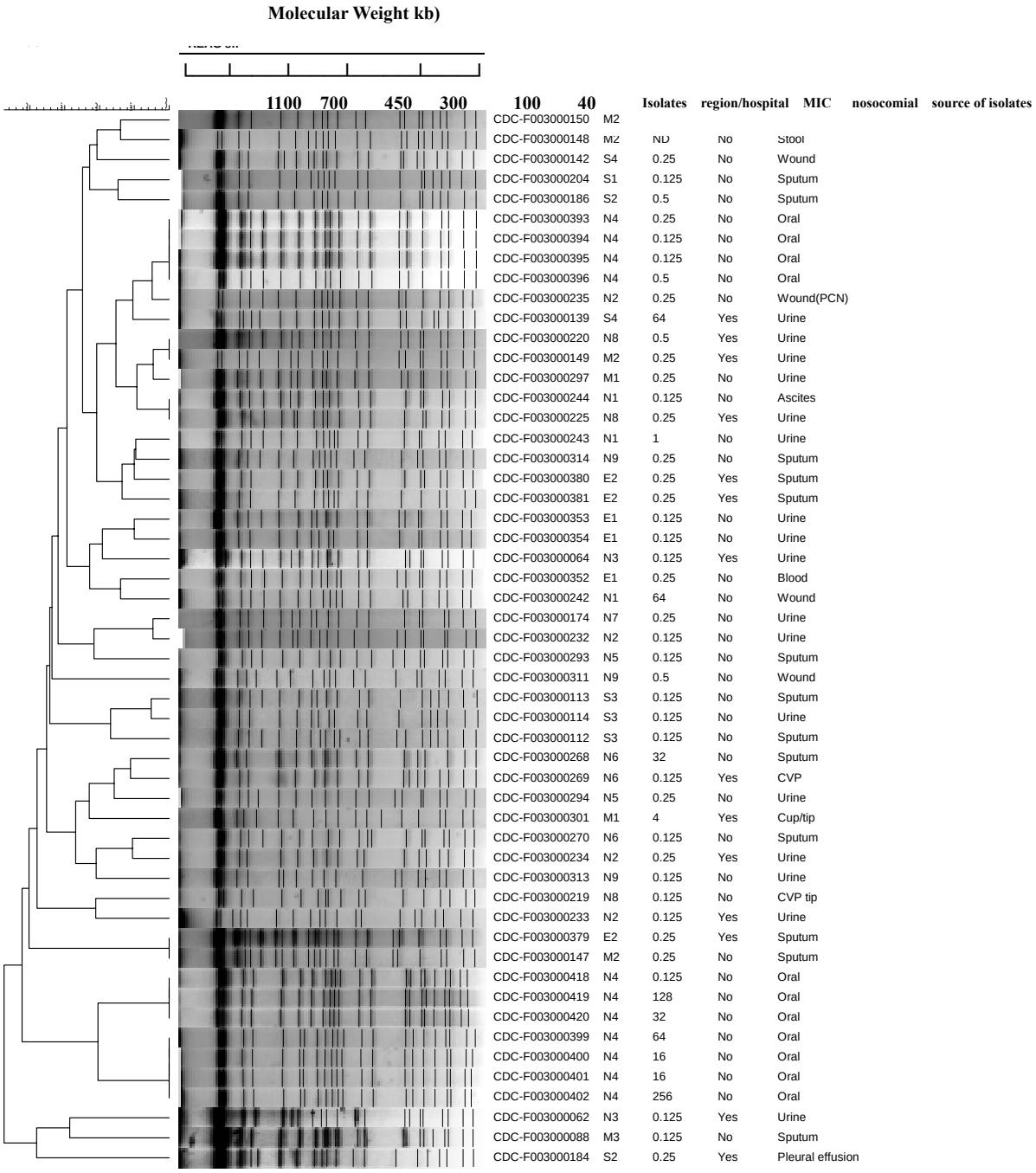


圖二、 PCR-EIA 和 PCR 方法測試 *Candida* DNA 的敏感度。Lanes: M, molecular size markers GeneRuler^{TM} 50bp DNA Ladder; 所加 DNA 量從 lanes 1 至 11 分別為 5 ng, 1 ng, 500 pg, 100 pg, 50 pg, 10 pg, 5 pg, 1pg, 500 fg, 100 fg, 50 fg, 10 fg。Lane 12 為陰性對照使用水取代 template DNA gave 並無任何反應產物。增幅產物以跑 agarose gel 檢測，產物也同時用 EIA 檢測。DNA 濃度以 A_{650} 值量測。結果依閥值判定為陽性或陰性分別以 + 或 − 表示。閥值（cut-off, 在此為 0.076）是取陰性對照平均值加二倍標準偏差。

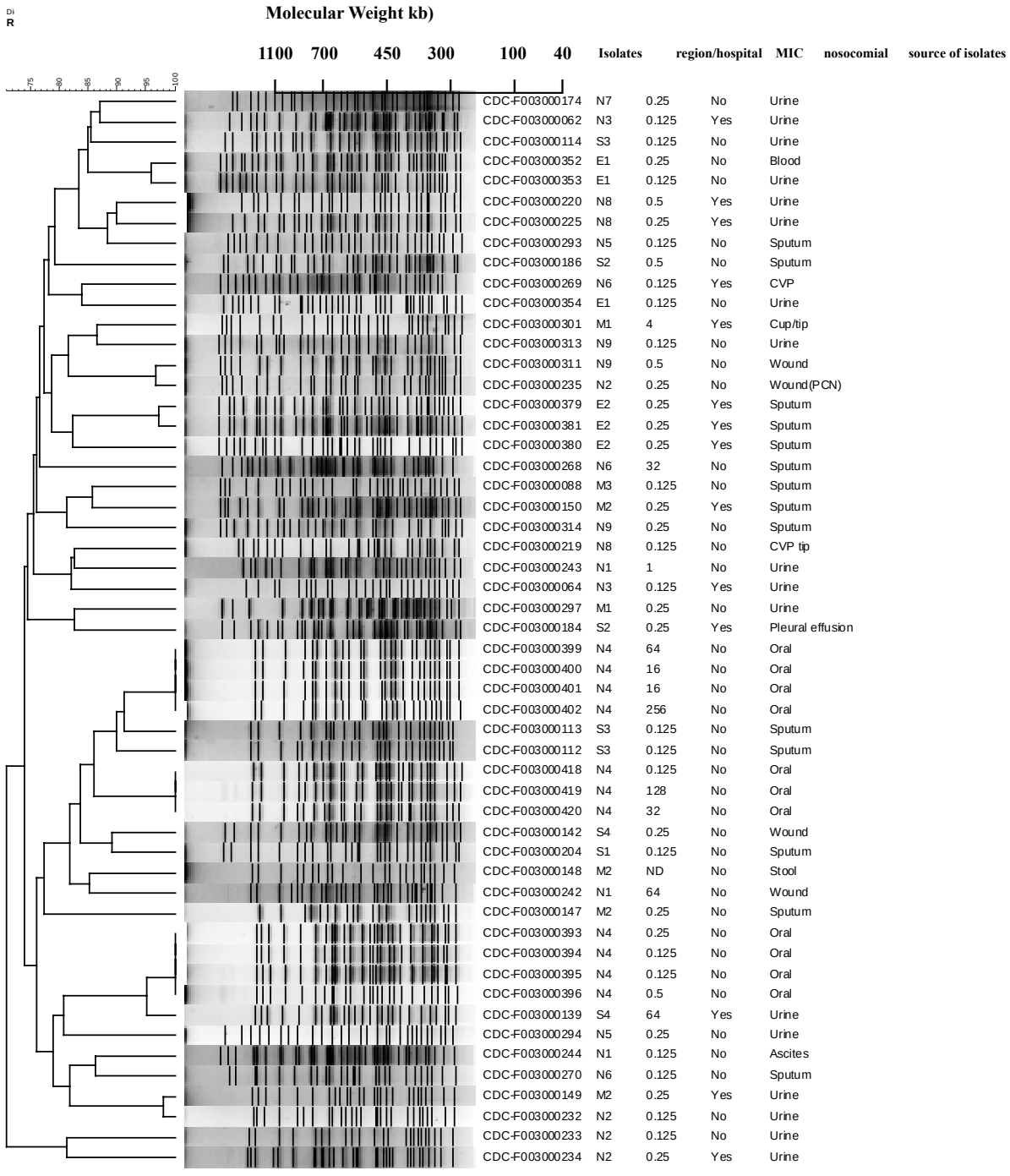
圖三、以PFGE-karyotyping 分子分型法分析53株*C. albicans*臨床菌株。每個 HIV 病人以方框標示。地區: N, 北; S, 南; M, 中; E, 東. MIC 代表 每株菌的 fluconazol 抗藥性。ND, 未測。



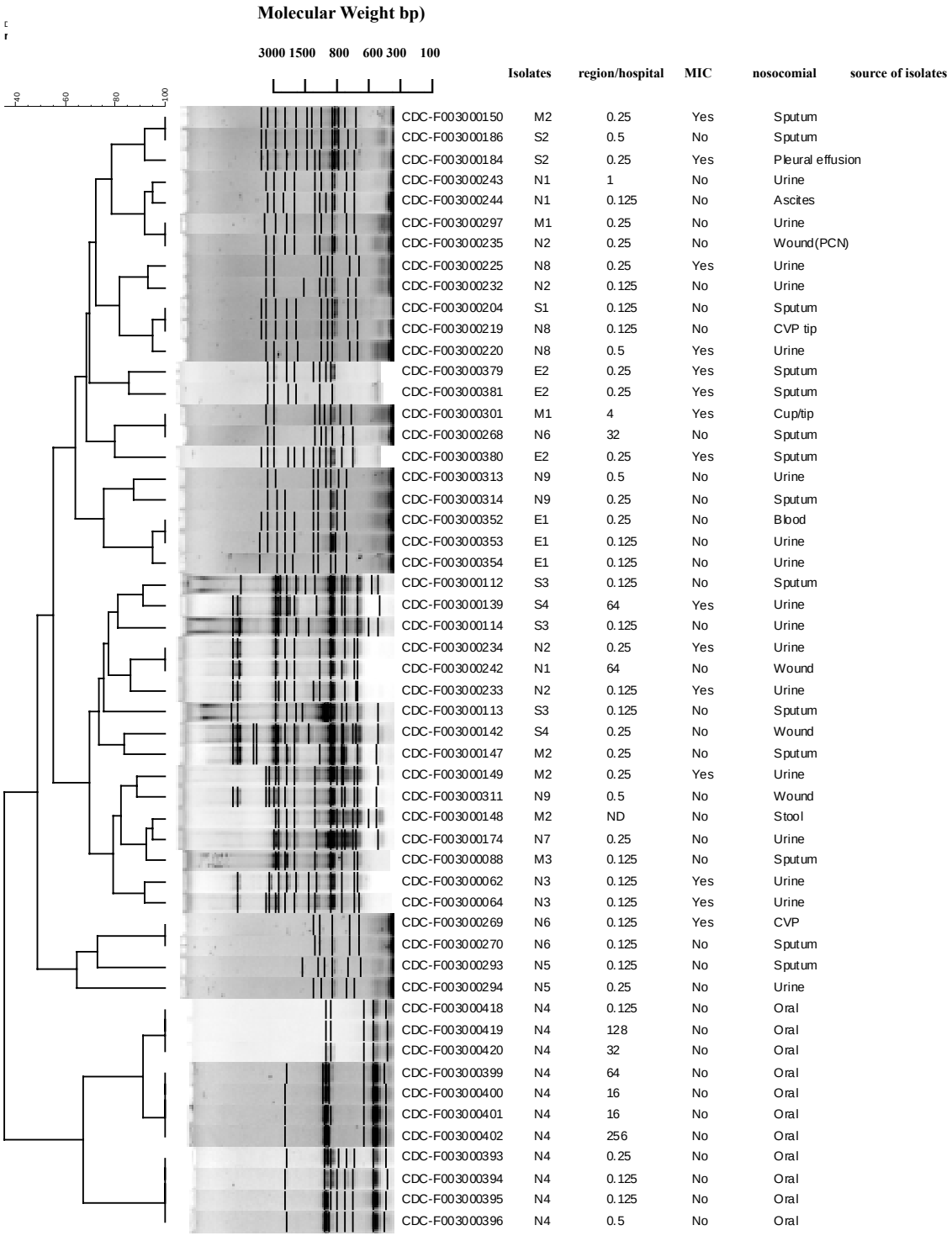
圖四、以PFGE-*Sfi*I 分子分型法分析53株*C. albicans*臨床菌株。每個 HIV 病人以方框標示。地區: N, 北; S, 南; M, 中; E, 東. MIC 代表 每株菌的 fluconazol 抗藥性。ND, 未測。



圖五、以PFGE-*Bss*HII分子分型法分析53株*C. albicans*臨床菌株。每個HIV病人以方框標示。地區: N, 北; S, 南; M, 中; E, 東. MIC 代表 每株菌的 fluconazol 抗藥性。 ND, 未測。



圖六、以rep-PCR分子分型法分析53株*C. albicans*臨床菌株。每個 HIV 病人以方框標示。地區: N, 北; S, 南; M, 中; E, 東. MIC 代表 每株菌的 fluconazol 抗藥性。ND, 未測。



Isolates	region/hospital	MIC	nosocomial	source of isolates
CDC-F003000150	M2	0.25	Yes	Sputum
CDC-F003000186	S2	0.5	No	Sputum
CDC-F003000184	S2	0.25	Yes	Pleural effusion
CDC-F003000243	N1	1	No	Urine
CDC-F003000244	N1	0.125	No	Ascites
CDC-F003000297	M1	0.25	No	Urine
CDC-F003000235	N2	0.25	No	Wound(PCN)
CDC-F003000225	N8	0.25	Yes	Urine
CDC-F003000232	N2	0.125	No	Urine
CDC-F003000204	S1	0.125	No	Sputum
CDC-F003000219	N8	0.125	No	CVP tip
CDC-F003000220	N8	0.5	Yes	Urine
CDC-F003000379	E2	0.25	Yes	Sputum
CDC-F003000381	E2	0.25	Yes	Sputum
CDC-F003000301	M1	4	Yes	Cup/tip
CDC-F003000268	N6	32	No	Sputum
CDC-F003000380	E2	0.25	Yes	Sputum
CDC-F003000313	N9	0.5	No	Urine
CDC-F003000314	N9	0.25	No	Sputum
CDC-F003000352	E1	0.25	No	Blood
CDC-F003000353	E1	0.125	No	Urine
CDC-F003000354	E1	0.125	No	Urine
CDC-F003000112	S3	0.125	No	Sputum
CDC-F003000139	S4	64	Yes	Urine
CDC-F003000114	S3	0.125	No	Urine
CDC-F003000234	N2	0.25	Yes	Urine
CDC-F003000242	N1	64	No	Wound
CDC-F003000233	N2	0.125	Yes	Urine
CDC-F003000113	S3	0.125	No	Sputum
CDC-F003000142	S4	0.25	No	Wound
CDC-F003000147	M2	0.25	No	Sputum
CDC-F003000149	M2	0.25	Yes	Urine
CDC-F003000311	N9	0.5	No	Wound
CDC-F003000148	M2	ND	No	Stool
CDC-F003000174	N7	0.25	No	Urine
CDC-F003000088	M3	0.125	No	Sputum
CDC-F003000062	N3	0.125	Yes	Urine
CDC-F003000064	N3	0.125	Yes	Urine
CDC-F003000269	N6	0.125	Yes	CVP
CDC-F003000270	N6	0.125	No	Sputum
CDC-F003000293	N5	0.125	No	Sputum
CDC-F003000294	N5	0.25	No	Urine
CDC-F003000418	N4	0.125	No	Oral
CDC-F003000419	N4	128	No	Oral
CDC-F003000420	N4	32	No	Oral
CDC-F003000399	N4	64	No	Oral
CDC-F003000400	N4	16	No	Oral
CDC-F003000401	N4	16	No	Oral
CDC-F003000402	N4	256	No	Oral
CDC-F003000393	N4	0.25	No	Oral
CDC-F003000394	N4	0.125	No	Oral
CDC-F003000395	N4	0.125	No	Oral
CDC-F003000396	N4	0.5	No	Oral

圖七、 28 株 *C. albicans* 臨床菌株及 2 株標準菌株變異的核甘酸位置和基因座(locus)上的各種 alleles。各欄顯示有多型性的位置。因為 *C. albicans* 為雙倍體變異代碼: K, G 或 T; M, A 或 C; R, A 或 G; S, C 或 G; W, A 或 T; Y, C 或 T. 括號內的數目代表有這種 allele 的數目。

CaACC1 Alleles	218	211	281	317	319
1(19)	G	A	C	C	C
2(1)	R				
3(1)		C		T	
4(1)		M			
5(1)		M		Y	
6(4)			T		
7(1)			Y		
8(1)			Y		Y
9(1)				Y	

CaGLN4 Alleles	675	757	795	127	238	391	393
1(4)	T	T	T	G	T	A	A	C
2(1)				R				
3(9)			W	R				
4(2)			A	A	Y			
5(5)			W	A				
6(1)			A	A				
7(1)			W	R	Y			
8(1)		Y	W	R				
9(1)	Y	Y		A				
10(1)			W	R			M	M
11(1)				R		R	M	M
12(1)			A	A		R	M	M
13(1)			W	R		R	M	M
14(1)			W	R		R	C	

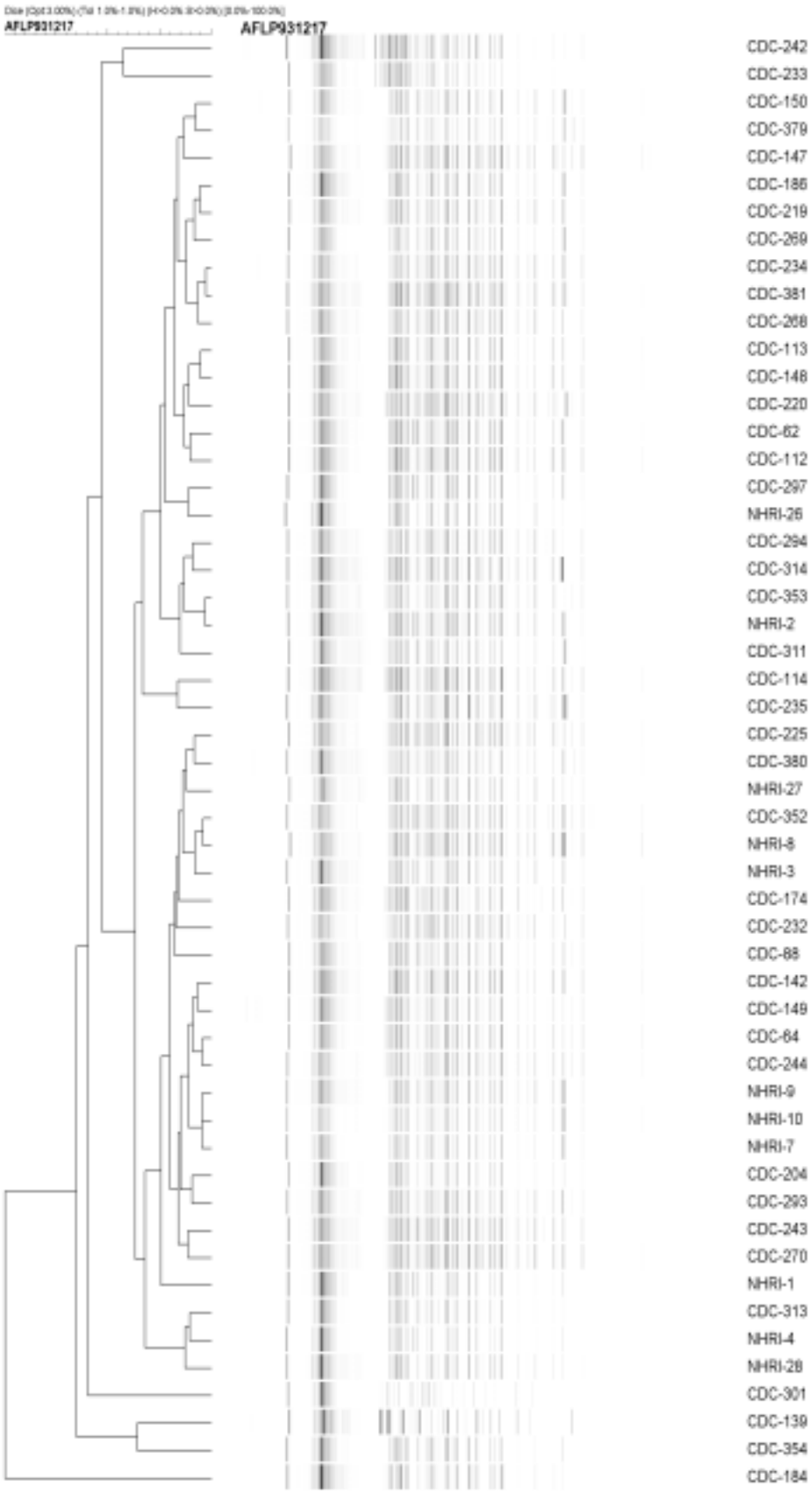
CaVPS13 Alleles	149	134	121	217	241	281	291	312	320	322	326	338	370
1(3)	C	A	G	T	A	A	G	C	G	T	G	G	T
2(3)		W								K			
3(4)										K	R		
4(1)				Y		R				K			
5(1)						R				K		S	W
6(3)				C		R							W
7(1)				Y		R							A
8(3)				Y		R							W
9(2)				Y		G						S	A
10(1)				Y				Y		K			A
11(1)				Y				Y		K			W
12(1)				Y		R				K			W
13(1)				Y	R	G	R						W
14(1)					R	R	R						W
15(1)			R			G			R				A
16(1)	M		R			G			R				A
17(1)						G	R		R				A
18(1)	M					R			R				W

CaADP1 Alleles	335	340	406	449	109	125	146	166	205	225	232	243	443
1(8)	C	A	C	C	A	A	A	T	G	G	T	C	A	A
2(4)			Y	Y		R	R		R	R	W	Y		
3(1)			T	T		G	G		A	A	A	T		
4(1)			T	T		G	R		A	A	A	T		
5(1)	G		T	T		G	G		A	A	A	T		
6(1)			Y	Y		R	R		R	R	A	Y		
7(1)			Y	Y		R	G		R	R	W	Y	M	W
8(1)				Y	W	R	R	Y	R	R	A	Y		
9(3)		G												
10(8)		R												
11(1)				Y		R	R		R	R	W	Y		

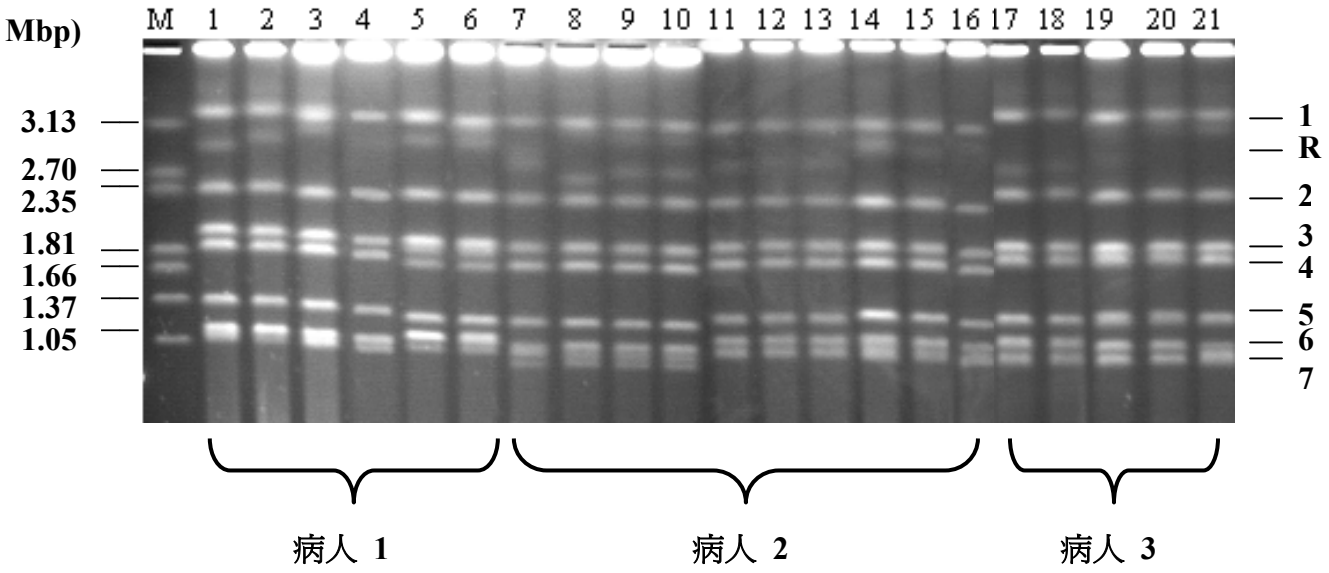
CaRPN2 Alleles	19	34	36	46	49	55	76	106	130	142	162	274
1(2)	G	T	T	A	A	G	G	A	G	A	G	C
2(2)	T				G	A	A					
3(2)	K				R	R	R					S
4(1)	K	W			R	R	R					
5(1)	K				R	R	R				R	S
6(1)	K		R		R	R	R			R		
7(2)												S
8(7)												G
9(3)				R					R			S
10(1)		W	Y									S
11(4)				G						G		
12(3)				R						R		
13(1)	K			R				R		R		

CaSYA1 Alleles	1	25	61	100	122	142	160	185	307	328
1(3)	T	C	A	T	C	G	C	G	C	T
2(1)	Y		R	C		A		K		
3(1)	C		A	C		A		T		
4(4)	Y	M	R	C		A		K		
5(1)	Y	M	R	C		A	Y	K		
6(2)	Y	M	R	C	M	A	Y	K		
7(3)		A	G	C		A	T	K		
8(1)		A	R	C		A	Y	K		
9(1)		A	R	C		A	T	K		
10(1)		M		C		A	T			
11(1)	Y	M	R	Y		R	Y	K		
12(1)	Y		R							
13(2)									A	
14(2)		M							A	
15(2)	Y	M	R							M
16(1)	C		G							
17(1)	Y		R	Y		R				
18(1)	Y	M	R	Y		R		K		
19(1)	Y		R	Y		R		K		Y

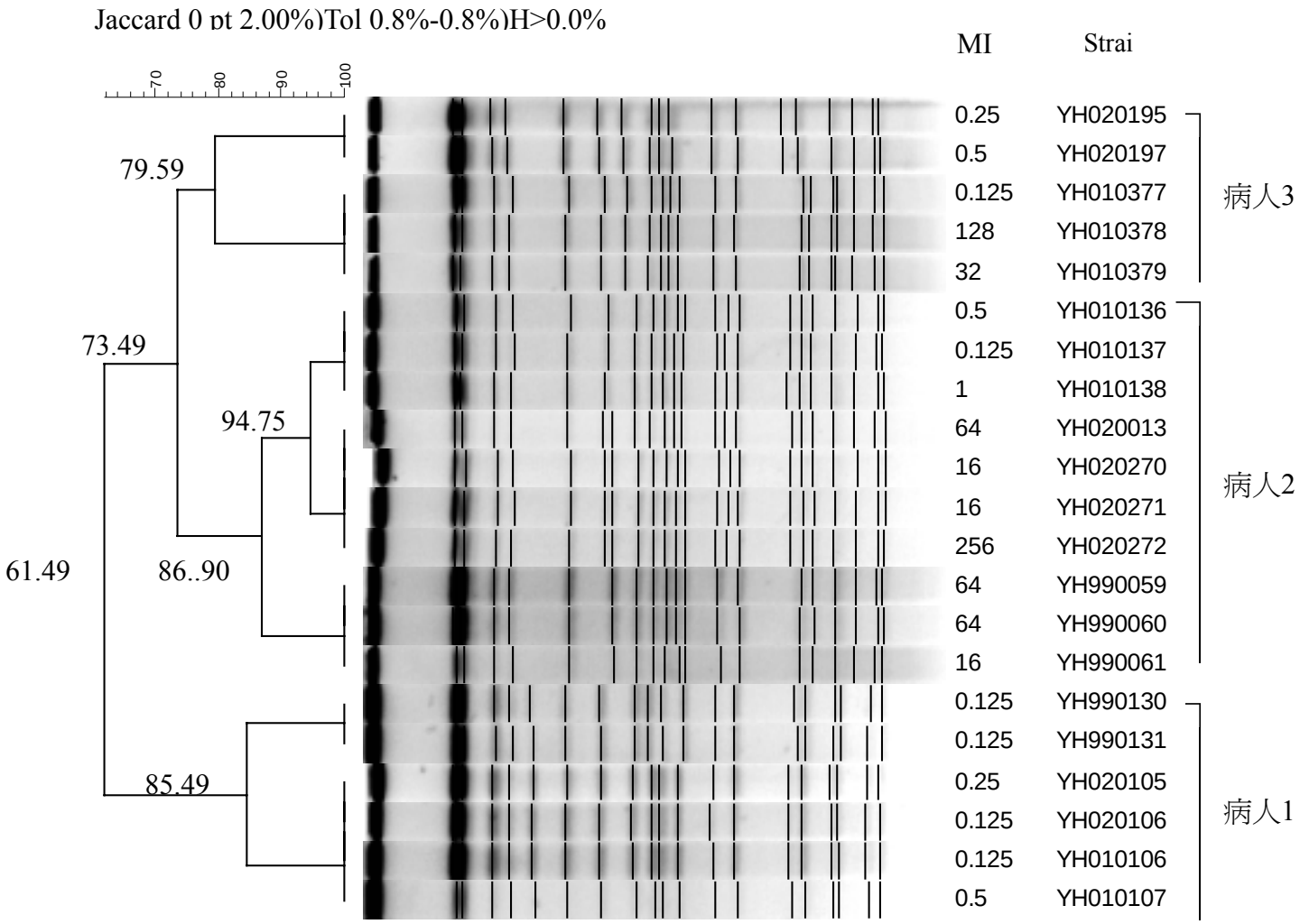
圖八、AFLP分析21株*C. albicans* 臨床菌株間之類緣關係。



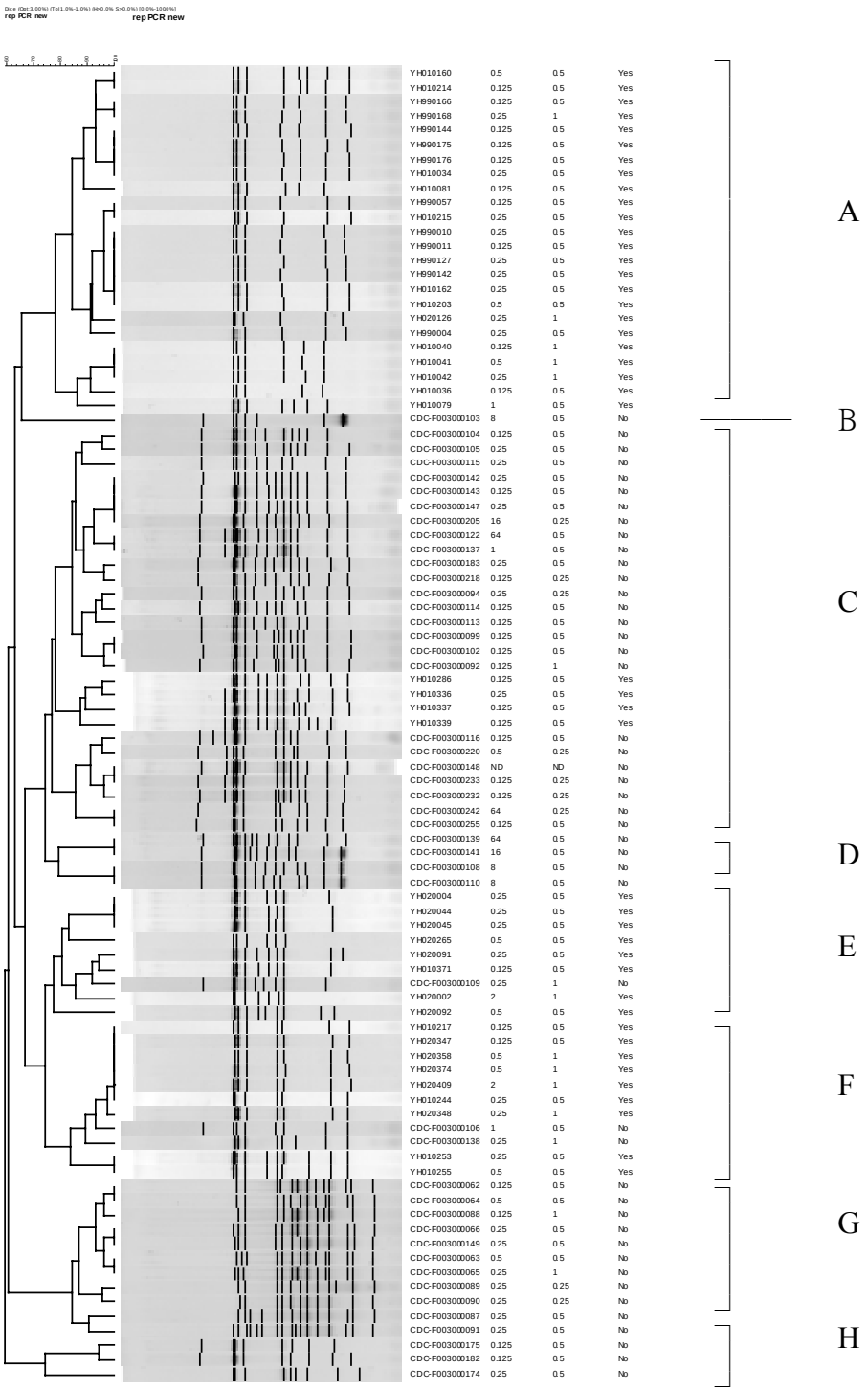
圖九、21 株 *C. albicans* 臨床菌株之核型。lanes 1 至 6 為病人#1 的檢體, lanes 7 至 16 為病人#2 的檢體, lanes 17 至 21 為病人#3 的檢體。1 至 21 菌株編號依序為:CDC-F003000393, CDC-F003000394, CDC-F003000395, CDC-F003000396, CDC-F003000397, CDC-F003000398, CDC-F003000399, CDC-F003000400, CDC-F003000401, CDC-F003000402, CDC-F003000403, CDC-F003000404, CDC-F003000405, CDC-F003000406, CDC-F003000407, CDC-F003000408, CDC-F003000420, CDC-F003000416, CDC-F003000417, CDC-F003000418,和 CDC-F003000419。M 為以 *Hansenula wingei* 菌的酵素切割片段做分子量標準。



圖十、PFGE- *Sfi* I 分析 21 株 *C. albicans* 臨床菌株間之類緣關係。樹狀圖以 BioNumerics 軟體建立, 條件用 2% optimization 和 0.8%的位置容忍度,依據 Jaccard similarity coefficients 計算 UPGMA 算數平均。.



圖十一、rep-PC分析21株*C. albicans* 臨床菌株間之類緣關係。



八、附錄：本計畫產出著作發表成果

i. SCI 論文

Kuo-Wei Chen, Hsiu-Jung Lo, Tzu-Hui Wang, Yu-Hui Lin, Shu-Ying Li (2005) Comparison of Four Molecular Typing Methods to Assess the Genetic Relatedness of Candida albicans Clinical Isolates in Taiwan. J. Med. Microbiol. 付印中.

ii. SCI 論文(與國衛院合作成果)

Yun-Liang Yang, Shu-Ying Li, Hsiao-Hsu Cheng, Hsiu-Jung Lo, TSARY Hospitals (2004) Susceptibilities to amphotericin B 和 fluconazole of Candida species in TSARY 2002. Diagn. Micr. Infec. Dis. 2004 年 12 月付印.

iii. 疫情報導

林于勤,李淑英,陳豪勇,林鼎翔 2004 新型恆溫式圈環形核酸增幅法簡介與應用—Loop-mediated Isothermal Amplification（LAMP）,疫情報導 2004, 206321-330.

iv. 已投寄至 SCI 期刊論文

Shu-Ying Li, Yun-Liang Yang, Kuo-Wei Chen, Hsiao-Hsu Cheng, Chien-Shun Chiou, Tzu-Hui Wang, Tsai-Ling Launderdale, Chen-Ching Hung, Hsiu-Jung Lo (2004) Molecular Epidemiology of Long-term Colonization of Candida albicans Strains from HIV-infected Patients (2003年12月已投寄).

v. 將投寄至 SCI 期刊論文

Kuo-Wei Chen, Yee-Chun Chen, Hsiu-Jung Lo, Tzu-Hui Wang, Hsiao-Hui Chou, Shu-Ying Li (2005) Multilocus Sequence Typing of Geographically Diverse Candida albicans strains in Taiwan.

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Comparison of four molecular typing methods to assess genetic relatedness of *Candida albicans* clinical isolates in Taiwan

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This report describes the investigation of the genetic profiles of 53 *Candida albicans* isolates collected from 18 hospitals in Taiwan using three PFGE-based typing methods (PFGE karyotyping, and PFGE of *Sfi*I and *Bss*HII restriction fragments) and one repetitive-sequence-PCR (rep-PCR) method. All four methods were able to identify clonal related isolates from the same patients. PFGE-*Bss*HII exhibited the highest discriminatory power by discriminating 40 genotypes, followed by PFGE-*Sfi*I (35 genotypes) and then by rep-PCR (31 genotypes), while PFGE karyotyping exhibited the lowest discriminatory power (19 genotypes). High discriminatory power can also be achieved by combining typing methods with different typing mechanisms, such as rep-PCR and PFGE-based typing methods. The results also showed that the genotype of each isolate was patient-specific and not associated with the source of the isolation, geographic origin or antifungal resistance.

INTRODUCTION

In recent decades, the incidence of invasive *Candida* infections has increased and has emerged as an important public health problem (Hajjeh *et al.*, 2004; Tortorano *et al.*, 2003). *Candida albicans* accounts for more than 50 % of systemic candidiasis and is the most pathogenic *Candida* species (Pfaller *et al.*, 2000). Since 2001, *Candida* species and other yeasts have ranked as the third most frequent nosocomial infection in Taiwanese medical centres, with *C. albicans* being the leading cause of yeast infections (Chen *et al.*, 2003).

Molecular typing methods are increasingly used in the analysis of strain relatedness in order to identify transmission routes (Elias Costa *et al.*, 1999), decide on prophylaxis practices (Chen *et al.*, 2001) and assess the biodiversity of a microbial population (Clemons *et al.*, 1997). Molecular typing methods based on different principles have been developed to fulfil such purposes. These methods include RFLP of the whole genome by using *Eco*RI, *Hinf*I and *Msp*I restriction enzymes (Poikonen *et al.*, 2001), RFLP followed by probe hybridization (Taylor *et al.*, 2003), PFGE karyotyping, PFGE of fragments generated by rare-cutting restriction endonucleases such as *Sfi*I (Kanellopoulou *et al.*, 2001), *Sma*I, *Not*I and *Bss*HII (Riederer *et al.*, 1998), PCR fingerprinting of the whole genome by randomly amplified polymorphic DNA (RAPD) analysis (Samaranayake *et al.*, 2003) or amplified fragment length polymorphism (AFLP) analysis (Ball *et al.*, 2004), fingerprinting of repetitive sequences by repetitive-sequence-based PCR (rep-PCR; Redkar *et al.*, 1996) and sequencing of house-keeping genes by multi-locus sequence typing (MLST; Bougnoux *et al.*, 2003). Molecular typing methods should be reproducible, discriminatory, high throughput, easy-to-use, digitally portable and amenable to standardization and library typing (Soll, 2000). For more than two decades, PFGE-based typing methods have been widely used and shown to be discriminatory and reproducible. The method of rep-PCR represents a more convenient PCR-based genomic fingerprinting technique; it uses primers directed to the interspersed repetitive DNA elements present at certain characteristic locations in the genome. Rep-PCR has the advantage of being labour-saving, rapid and economical.

In this paper, we used three PFGE-based typing methods (PFGE karyotyping, *Sfi*I-PFGE and *Bss*HII-PFGE) and a rep-PCR typing method to study 53 clinical isolates of *C. albicans* collected from 18 hospitals in Taiwan. The aims of this study were to compare the discriminatory power of these four molecular typing methods and to ascertain whether different characteristics (e.g. drug resistance, geographic origin, source of isolate, nosocomial or not) could be attributed to certain specific molecular types in Taiwan. The most discriminatory typing method will be standardized and serve as

Abbreviations: AFLP, amplified fragment length polymorphism; DI, discriminatory index; MLST, multi-locus sequence typing; RAPD, randomly amplified polymorphic DNA; rep-PCR, repetitive-sequence-PCR.

the tool for future outbreak investigations and the basis for comparison of other typing methods. The data obtained in this study will also contribute to our attempt to establish a central genetic database of fungal pathogens and provide a platform for the comparison of domestic as well as international fungal genotypes.

METHODS

Fungal strains. A total of 53 *C. albicans* clinical isolates were used in this study. Information on each isolate was collected, including the MIC of fluconazole, geographic origin and body site origin, as well as whether it was a nosocomial infection. Nosocomial infections were identified by hospital infection control practitioners in the course of routine surveillance according to the criteria of the Center for Disease Control and Prevention (CDC) (Garner *et al.*, 1988).

Forty-two isolates were part of the collections of the Taiwan Surveillance of Antimicrobial Resistance of Yeasts Project, which collected clinical strains isolated from 22 hospitals located in different geographic regions in Taiwan from April 15 to June 15 1999 (Yang *et al.*, 2004). Only one isolate was accepted during each episode of infection. The remaining eleven isolates (CDC-F003000393–CDC-F003000396, CDC-F003000399–CDC-F003000402, CDC-F003000418–CDC-F003000420) were serial oral isolates collected from three HIV-infected patients between 2001 and 2002. The three HIV patients were outpatients from the same hospital.

The identification of all fungal strains was undertaken by the germ-tube assay followed by VITEK Yeast Biochemical Card and API-32C systems. The MIC for fluconazole of the *C. albicans* isolates were determined by the microdilution broth method according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) document M27-A, as described previously (Yang *et al.*, 2003).

PFGE karyotyping. Strains were inoculated on Sabouraud dextrose agar (SDA; Difco) for 48 h at 37 °C. Colonies on agar were picked in a cell suspension buffer (100mM Tris/HCl, pH 8·0; 100 mM EDTA, pH 8·0). The cell density of the suspension for the plug was estimated by measuring the OD_{600} and adjusting to OD_{600} 1·5. The cells were pelleted by centrifugation at 3000 *g* for 5 min and resuspended in 500 µl of cell suspension buffer. Then, 100 µl of lyticase (Sigma; 1250 unit ml^{-1} in 50 % glycerol and 0·01 M N_3PO_4) was added to the suspension, which was incubated at 37 °C for 30 min. Next, 600 µl of 1 % (w/v) agarose (Seakem Gold agarose; BioWhittaker Molecular Applications) in TE buffer (10 mM Tris/HCl, pH 8·0; 1 mM EDTA, pH 8·0) was added. After mixing, the solution was poured into the wells of plug moulds and kept at room temperature for 5 min for solidification. The plugs were transferred into 50 ml tubes containing 5 ml of cell lysis buffer (100 mM Tris/HCl, pH 8·0, 0·45 M EDTA, pH 8·0, 1 % *N*-lauroylsarcosine, 1 mg proteinase K ml^{-1}) and incubated overnight in a shaker water bath at 50 °C. The plugs were washed twice with double-distilled H_2O at 50 °C for 15 min and TE buffer at 50 °C for 10 min and then stored in TE buffer until use.

Electrophoresis was performed with a Biometra Rotaphor at pulse time 60–700 s, angle 120°, 120–90 V in 0·8 % agarose gel with 0·5× TBE (50 mM Tris, 45 mM boric acid, 0·5 mM EDTA) for 66 h. After electrophoresis, the gel was stained with ethidium bromide solution for 15 min and destained with distilled water. DNA fragments were imaged with IS-1000 Digital Imaging System (Alpha Innotech).

PFGE of *Sfi*I and *Bss*HII restriction fragments. The plugs were cut into 2-mm-wide slices. For *Sfi*I digestion, the slices were placed in 200 µl of buffer 2 solution (50 mM NaCl, 10 mM Tris/HCl, 10 mM $MgCl_2$, 1 mM DTT) containing 2 µl of BSA (New England BioLabs) and incubated for 1 h at 50 °C. The plug slices were transferred to 200 µl of buffer 2 solution containing 2 µl of BSA and 20 units of *Sfi*I and incubated at 50 °C overnight. For *Bss*HII digestion, the slices were placed in 200 µl of buffer 3 solution (100 mM NaCl, 50 mM Tris/HCl, 10 mM $MgCl_2$, 1 mM DTT) (New England BioLabs) and incubated for 1 h at 50 °C. The plug slices were transferred to 200 µl of buffer 3 solution containing 4 units of *Bss*HII and incubated at 50 °C overnight. Electrophoresis was performed with a Biometra Rotaphor at pulse time 6–50 s, angle 120°, 180 V in 0·8 % agarose gel with 0·5× TBE for 36 h. After electrophoresis, the gel was stained with ethidium bromide solution for 15 min and destained with distilled water.

rep-PCR. The total genomic DNA of the strain was extracted by using PUREGENE DNA Purification Kit (Gentra) as was described previously (Hsu *et al.*, 2003). The concentration of DNA extracted from *C. albicans* isolates was measured with a spectrophotometer (A_{260}). DNA was stored at −80 °C until use.

The rep-PCR reaction was performed by using primers Ca-21 (5′-CATCTGTGGTGGAAAGTTAAC-3′) and Ca-22 (5′-ATAATGCT CAAAGGTGGTAAG-3′) designed from Care-2 repetitive elements and amplified variable regions between Care-2 elements as described previously, with some modifications (Redkar *et al.*, 1996). Reaction mixtures (20 µl) consisted of 10 mM Tris/HCl (pH 9·0), 50 mM KCl, 2·5 mM $MgCl_2$, 0·1 % Triton X-100, 0·2 mM dNTP mix, 50 pmol primer and 0·5 units of *Taq* polymerase. The amplification was performed by an initial denaturation at 95 °C for 5 min followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 42 °C for 1 min and extension at 72 °C for 2 min, and a final extension at 72 °C for 5 min in a Tpersonal thermocycler (Biometra). PCR products were analysed by electrophoresis through 1·5 % (w/v) agarose gel (Seakem LE agarose; Cambrex) in 1× TBE buffer. Lambda ladder 100–3000 bp was used as a DNA size standard. Gel electrophoresis was conducted in TBE buffer at 100 V cm^{-1} for 50 min. After electrophoresis, the gel was stained with ethidium bromide solution for 15 min and destained with distilled water.

Analysis of banding pattern. Dendrogram analysis was performed by using Bionumerics software version 3.0 (Applied Maths). Fingerprint similarity values were based on the presence or absence of bands between each profile pair being compared. The band inclusion window was adjusted by the size reference markers. Band assignment was done firstly by the automatic band search function of the software, then inspected visually to ensure that each assigned band had a characteristic densitometric profile of a Gaussian fit and had a corresponding band on the gel. Some weak bands resulted from incomplete digestion with restriction enzyme and were excluded manually. Incomplete digestion of DNA was observed for some isolates; intermediate bands, resulting from incomplete cutting, were confirmed by repeating the digestion of the DNA with a different amount of enzyme. The position tolerance was set at 1 % and optimization was set at 3 %. The Dice coefficient was used to analyse the similarities (S_{AB}) of the band patterns. The unweighted pair group method using arithmetic averages (UPGMA) was used for cluster analysis. Isolates were considered different when the band similarity value was less than 95 % (Voss *et al.*, 1998).

Calculation of discriminatory power. The discriminatory index (DI) of each of the four typing methods was determined by the application of Simpson's index (Hunter & Fraser, 1989). The DI is a measure of the probability that two unrelated strains sampled from the test population will be placed into different typing groups. A DI value of 1·0 would indicate that a typing method was able to distinguish each member of a strain population from all other members of that population. Conversely, a DI of 0·0 would indicate that all members of a strain population were of an identical type.

RESULTS AND DISCUSSION

The increasing frequency of invasive fungal infections and the high mortality rate associated with disseminated fungal diseases have underscored the importance of understanding the molecular epidemiology of fungal infections. We compared four molecular typing methods to ascertain their potential for outbreak investigation and to see whether different characteristics (drug resistance, geographic origin, source of isolation, nosocomial or not) could be attributed to certain specific molecular types in Taiwan. The data obtained in this study will serve as a tool for future outbreak investigations and a basis for comparison basis for other typing methods.

In this study, genetic profiles of 53 *C. albicans* clinical isolates from 45 patients were obtained and compared by PFGE karyotyping (Fig. 1), PFGE of *Sfi*I (Fig. 2) and *Bss*HII (Fig. 3) restriction fragments, and rep-PCR analysis (Fig. 4). All isolates were typable by all four typing methods. PFGE-*Bss*HII and -*Sfi*I generated 40 (DI = 0·995) and 35 (DI = 0·985) DNA patterns, respectively, for the 53 isolates, and were the most discriminatory in distinguishing isolates. Within the 42 isolates collected from different hospitals, 38 genotypes were found by using the PFGE-*Bss*HII method. The rep-PCR method revealed 31 distinct genotypes (DI = 0·983). PFGE karyotyping displayed seven- and eight-band patterns (excluding R chromosome) and was the least discriminatory method, generating 19 karyotypes (DI = 0·929) among the 53 strains analysed. When the R chromosome was considered in PFGE karyotyping, more patterns (25 types) were yielded (data not shown). However, the frequent size variation of R chromosome mainly stems from rRNA gene copy number differences and can alter within one cell division (Wickes *et al.*, 1991). Therefore, the dendrogram was constructed without analysing the R chromosome.

In contrast to the 42 isolates collected from different episodes, the year-long recurrent isolates from HIV patients served here as clonal/epidemiologically related isolates. All four methods were able to identify clonal related isolates from the same patients. Isolates from the same HIV patient belonged to one unique genotype. The 11 isolates collected from three HIV patients were assigned to three molecular types by all four typing methods. PFGE-restriction fragment methods yielded better results but the restriction endonuclease may vary by strains or species.

Ideally, a fingerprint pattern should comprise 20–30 bands within a wider molecular range. Restriction with the endonuclease *Not*I (Diaz-Guerra *et al.*, 1997) or *Sma*I (Doi *et al.*, 1994) generated around 10 bands in narrower molecular ranges, which was not sufficient. PFGE-*Sfi*I and -*Bss*HII, which recognize 13 and 6 bp restriction sites, respectively, provided more resolving power for the molecular typing of *C. albicans* infections. Restriction with the endonuclease *Sfi*I generated 18–22 (mean, 20) clear and well-separated fragments in the range of 40–1100 kb, which allowed clear differentiation with reasonable discriminatory power. PFGE-*Bss*HII resulted in more banding patterns (mean, 31 fragments; range 50–1000 kb) and provided the highest discriminatory power. Nevertheless, scoring PFGE-*Bss*HII banding patterns was more time-consuming than PFGE-*Sfi* I. An alternative would be to use *Sfi* I as the first typing enzyme, with the indistinguishable strains further typed by *Bss*HII. A combination of PFGE-*Sfi*I and -*Bss*HII methods resulted in a DI of 1·0.

For PFGE-based methods, band differences in DNA fingerprint profiles are primarily a result of polymorphisms in the recognition sites of individual restriction enzymes, translocation (Iwaguchi *et al.*, 2000) or reorganization of non-rDNA-containing chromosomes, or the non-reciprocal reorganization of rRNA gene cistrons in the rRNA-gene-containing chromosomes (Ramsey *et al.*, 1994). High concordance was found among the three PFGE-based methods (Table 1).

To our knowledge, no previous report has compared PFGE-based typing methods with the rep-PCR method for delineation of fungal strains. The PCR-based methods are less labour-intensive, and thus, potentially more suitable for routine use. A problem with RAPD typing using short and non-specific primers under low-stringency conditions is a lack of reproducibility (Dassanayake & Samaranayake, 2003). rep-PCR is chosen because it is based on an alternative typing mechanism, i.e. inter-repetitive sequence length polymorphism analysis, and is very simple and reproducible. Our data showed that PCR amplification of repetitive DNA elements in the *Candida* genome using the Ca-21 and Ca-22 primer pair generated simple patterns and offered less clustering and discriminatory power than PFGE with restriction enzymes. Low concordance was found between rep-PCR and the three PFGE-based methods (Table 1). However, when rep-PCR was used as a secondary typing method supplementary to electrokarytyping, PFGE-*Sfi*I or PFGE-*Bss*HII typing methods, high discriminatory indices of 0·996, 0·998 and 0·998, respectively, were obtained. The results highlight the usefulness of a combination of typing methods with different typing mechanisms.

All four methods used in this study were reproducible. This was demonstrated by the same DNA pattern being obtained in consecutive isolates from the same HIV patient and obtaining the same results by different experiments and in repeated runs.

Choosing appropriate molecular typing methods is essential for identifying the clonal relatedness of pathogens. The data obtained in this study will help to establish benchmarks for future surveillance in Taiwan, which will also serve as a basis for comparison of other typing methods.

Our results showed that the genotype of each isolate was patient-specific and no specific genotypes were predominant as to their ability to cause nosocomial infection or bloodstream infections. The panel of isolates used in this study were collected from 18 hospitals located around Taiwan and thus gave a good representation of diverse geographic origin. The

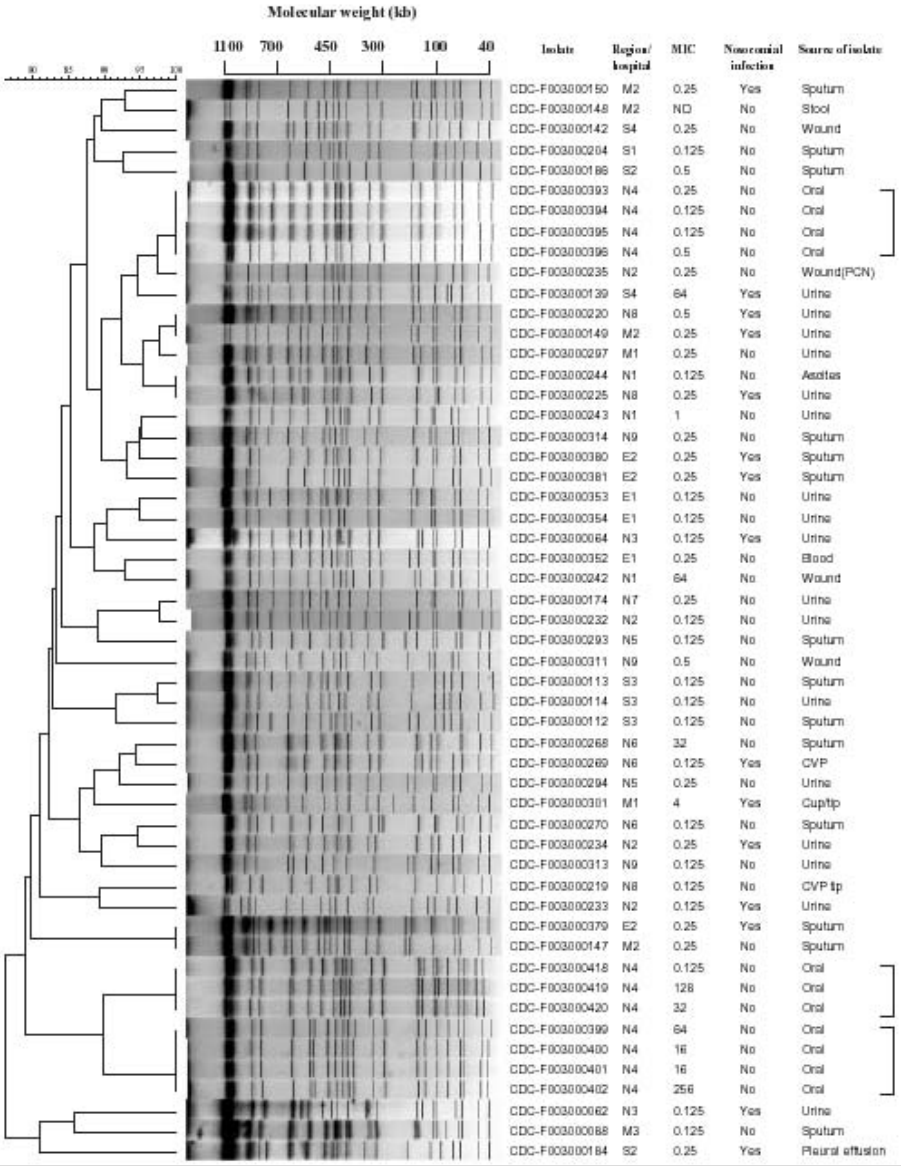

Fig. 1. Cluster analysis of the 53 *C. albicans* isolates based on the pattern of electrophoresis karyotype. Each of the three HIV patients was marked by a frame. Regions: N, north; S, south; M, middle; E, east. MIC indicates the MIC of fluconazol of each of the isolates. ND, Not determined.

extensive dispersion of genotypes among different hospitals illustrated that no correlation between genotypes and hospital/geographic origin could be established.

Monitoring genotype variations could have great implications for antifungal regimens. The five fluconazole-resistant strains (MIC > 64 μg ml^{-1}) were not clustered by any of the four typing methods (as shown in Figs 1, 2, 3 and 4) and the long-term recurrent isolates from each of the three HIV patients exhibited fluctuating fluconazole susceptibility/re-

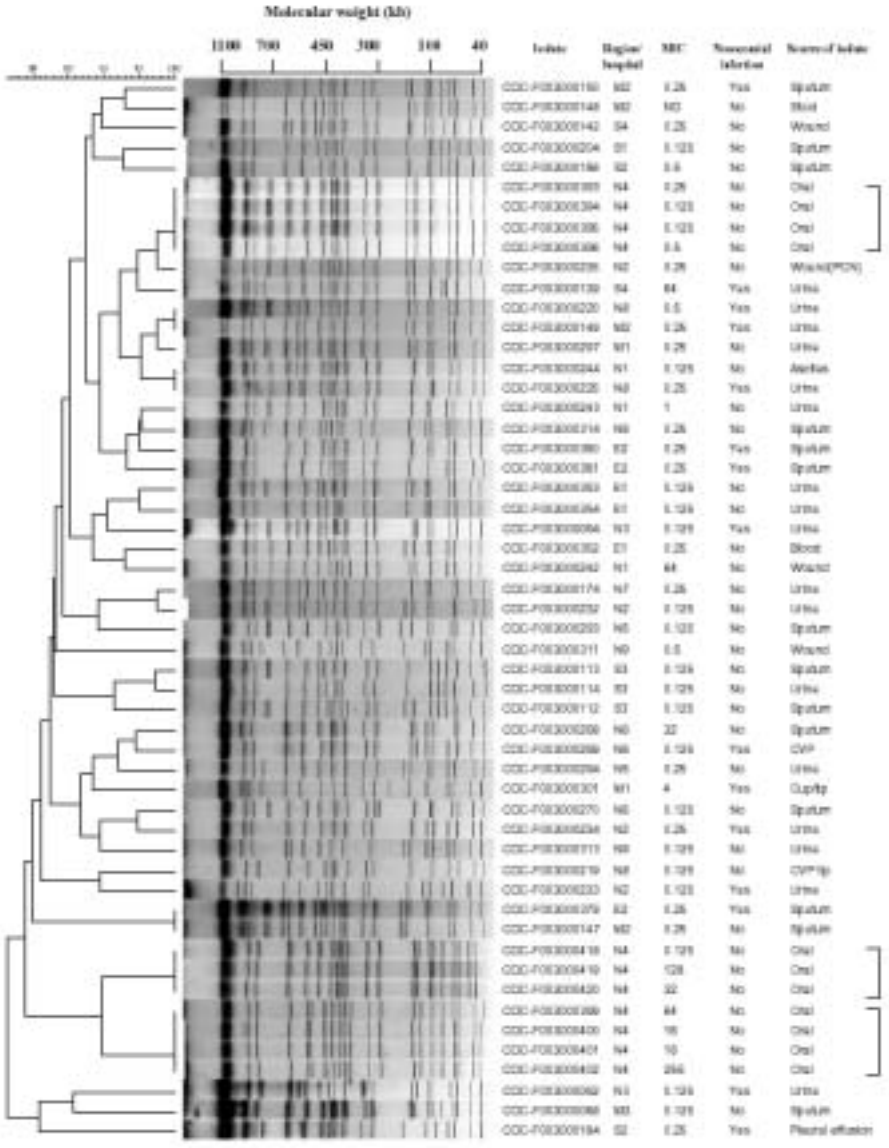

Fig. 2. Cluster analysis of the 53 *C. albicans* isolates based on the pattern of *Sfi*I restriction endonuclease analysis of genomic DNA. Each of the three HIV patients was marked by a frame. Regions: N, north; S, south; M, middle; E, east. MIC indicates the MIC of fluconazol of each of the isolates. nD, Not determined.

sistance levels but maintained the same genotype (Table 1). The scenario in our study is in concordance with many previous reports employing Ca3 repetitive element hybridization or RAPD methods (Dassanayake *et al.*, 2002; Lasker *et al.*, 2001; Makarova *et al.*, 2003). The difference in resistance to fluconazole is associated with subtle changes of genes involved in azole resistance (Franz *et al.*, 1998). For the detection of mutation, deletion or insertion events associated with drug

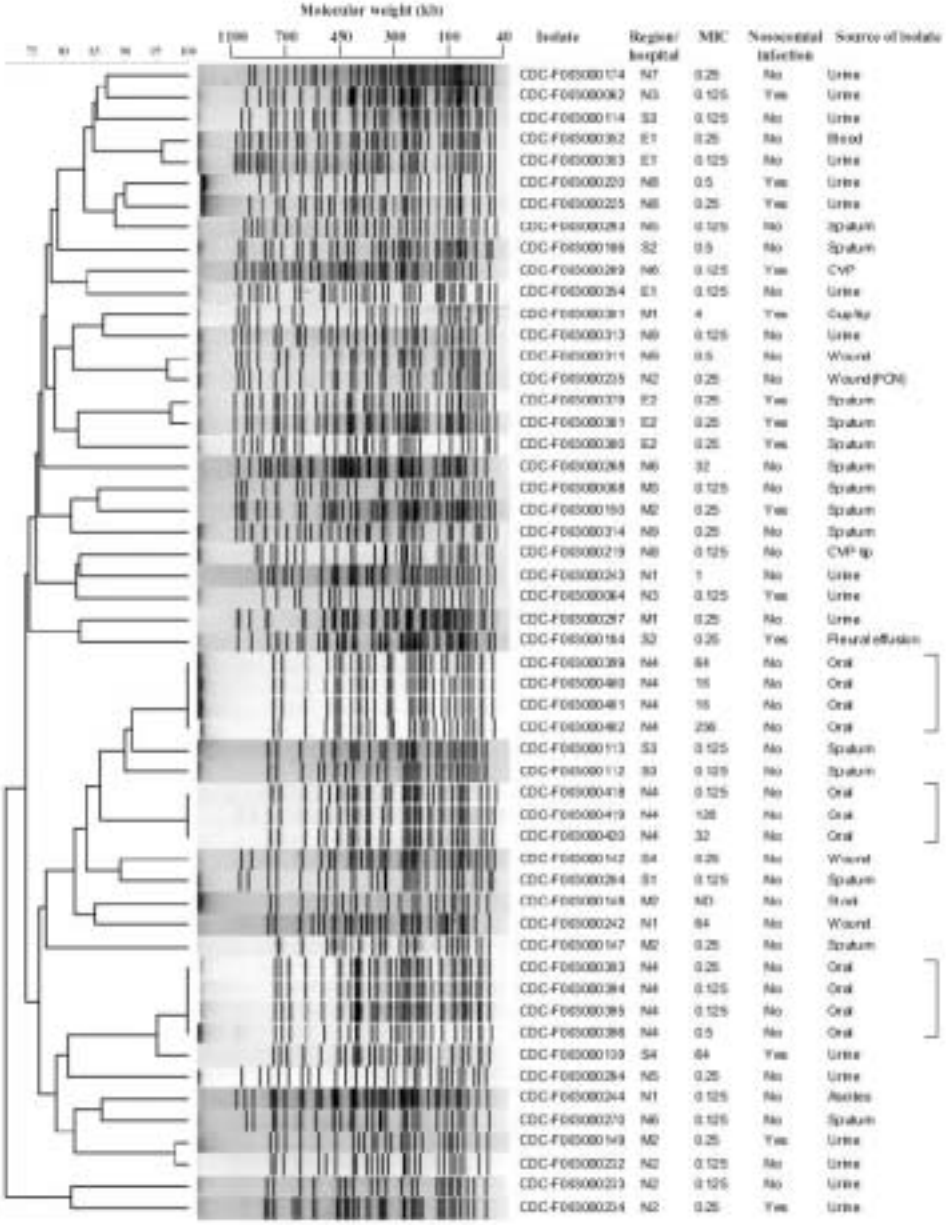

Fig. 3. Cluster analysis of the 53 *C. albicans* isolates based on the pattern of *Bss*HII restriction endonuclease analysis of genomic DNA. Each of the three HIV patients was marked by a frame. Regions: N, north; S, south; M, middle; E, east. MIC indicates the MIC of fluconazol of each of the isolates. ND, Not determined.

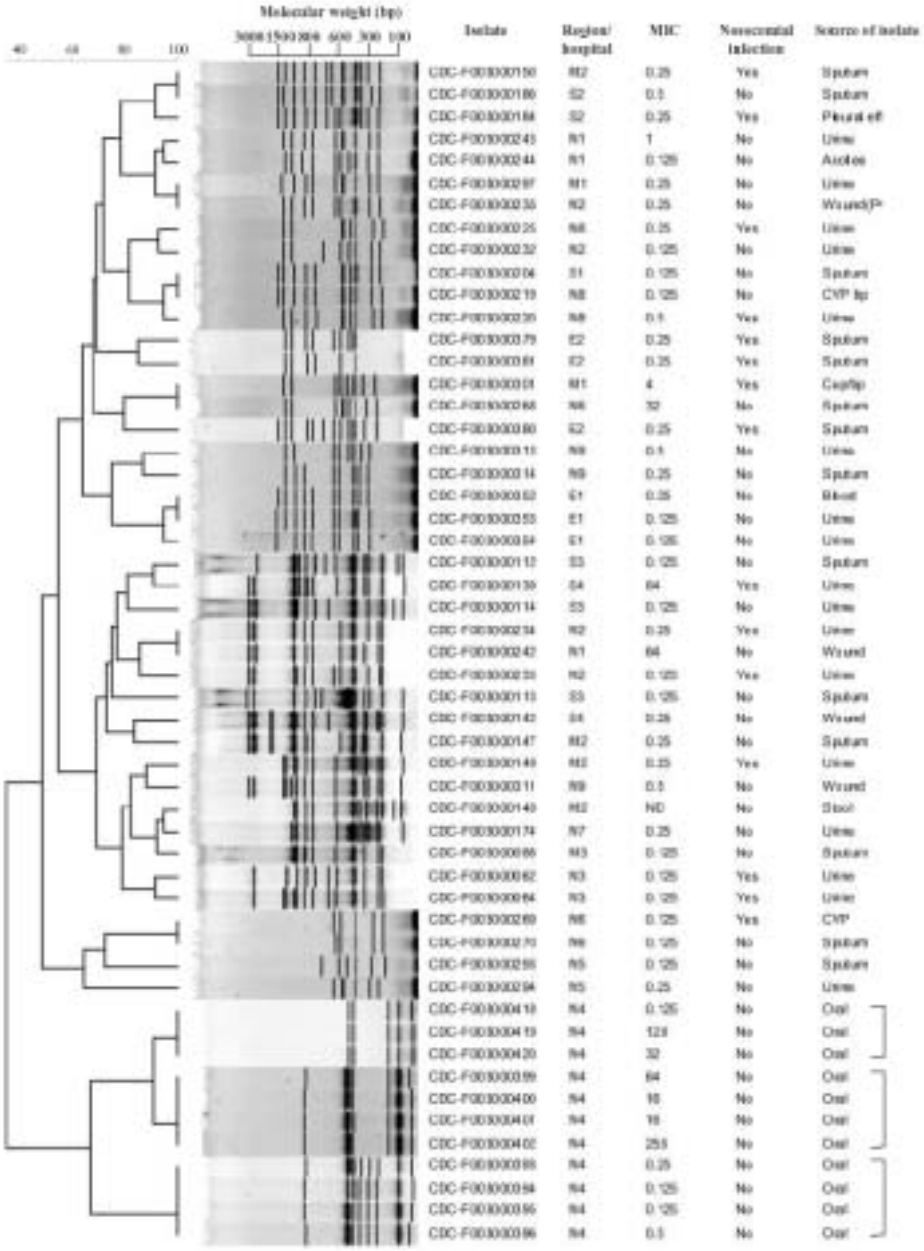

Fig. 4. Cluster analysis of the 53 *C. albicans* isolates based on the pattern of rep-PCR. Each of the three I–IV patients was marked by a frame. Regions: N, north; S, south; M, middle; E, east. MIC indicates the MIC of fluconazol of each of the isolates. ND, Not determined.

Table 1. Genotypes of 53 isolates of *C. albicans*

Code no.	PFGE karyotype (excluding R chromosome)	PFGE *SfiI*	PFGE *BssHII*	Rep PCR	Source of isolation*	Geological origin (region†/hospital)	Fluconazol MIC (μg ml^{-1})	Nosocomial infection	HIV status
CDC F003000062	A1	B1	C1	D1	Urine	N3	0·125	Yes	–
CDC F003000064	A1	B2	C2	D1	Urine	N3	0·125	Yes	–
CDC F003000088	A2	B3	C3	D2	Sputum	M3	0·125	No	–
CDC F003000112	A3	B4	C4	D3	Sputum	S3	0·125	No	–
CDC F003000113	A3	B5	C5	D4	Sputum	S3	0·125	No	–
CDC F003000114	A3	B5	C6	D5	Urine	S3	0·125	No	–
CDC F003000139	A4	B6	C7	D6	Urine	S4	64	Yes	–
CDC F003000142	A5	B7	C8	D7	Wound	S4	0·25	No	–
CDC F003000147	A6	B8	C9	D8	Sputum	M2	0·25	No	–
CDC F003000148	A7	B9	C10	D2	Stool	M2	ND	No	–
CDC F003000149	A8	B10	C11	D9	Urine	M2	0·25	Yes	–
CDC F003000150	A9	B11	C12	D10	Sputum	M2	0·25	Yes	–
CDC F003000174	A3	B12	C13	D2	Urine	N7	0·25	No	–
CDC F003000184	A10	B13	C14	D11	Pleural effusion	S2	0·25	Yes	–
CDC F003000186	A11	B14	C15	D10	Sputum	S2	0·5	No	–
CDC F003000204	A12	B15	C16	D12	Sputum	S1	0·125	No	–
CDC F003000219	A13	B16	C17	D12	CVP	N8	0·125	No	–
CDC F003000220	A8	B10	C18	D12	Urine	N8	0·5	Yes	–
CDC F003000225	A8	B10	C19	D13	Urine	N8	0·25	Yes	–
CDC F003000232	A3	B12	C20	D13	Urine	N2	0·125	No	–
CDC F003000233	A3	B17	C21	D14	Urine	N2	0·125	Yes	–
CDC F003000234	A14	B18	C22	D15	Urine	N2	0·25	Yes	–
CDC F003000235	A4	B6	C23	D16	Wound (PCN)	N2	0·25	No	–
CDC F003000242	A9	B19	C24	D15	Wound	N1	64	No	–
CDC F003000243	A8	B20	C11	D17	Urine	N1	1	No	–
CDC F003000244	A8	B10	C25	D17	Ascites	N1	0·125	No	–
CDC F003000268	A8	B21	C26	D18	Sputum	N6	32	No	–
CDC F003000269	A15	B22	C27	D19	CVP	N6	0·125	Yes	–
CDC F003000270	A7	B23	C28	D19	Sputum	N6	0·125	No	–
CDC F003000293	A4	B24	C29	D20	Sputum	N5	0·125	No	–
CDC F003000294	A16	B25	C30	D21	Urine	N5	0·25	No	–
CDC F003000297	A8	B10	C31	D16	Urine	M1	0·25	No	–
CDC F003000301	A17	B26	C32	D18	CVP	M1	4	Yes	–
CDC F003000311	A4	B27	C22	D22	Wound	N9	0·5	No	–
CDC F003000313	A18	B28	C33	D23	Urine	N9	0·125	No	–
CDC F003000314	A18	B29	C34	D24	Sputum	N9	0·25	No	–
CDC F003000352	A17	B30	C35	D25	Blood	E1	0·25	No	–
CDC F003000353	A17	B31	C35	D25	Urine	E1	0·125	No	–
CDC F003000354	A17	B31	C36	D25	Urine	E1	0·125	No	–
CDC F003000379	A6	B8	C37	D26	Sputum	E2	0·25	Yes	–
CDC F003000380	A17	B32	C38	D27	Sputum	E2	0·25	Yes	–
CDC F003000381	A6	B33	C37	D28	Sputum	E2	0·25	Yes	–
CDC F003000393	A4	B6	C7	D29	Oral	N4	0·25	No	+
CDC F003000394	A4	B6	C7	D29	Oral	N4	0·125	No	+
CDC F003000395	A4	B6	C7	D29	Oral	N4	0·125	No	+
CDC F003000396	A4	B6	C7	D29	Oral	N4	0·5	No	+
CDC F003000399	A8	B34	C39	D30	Oral	N4	64	No	+
CDC F003000400	A8	B34	C39	D30	Oral	N4	16	No	+
CDC F003000401	A8	B34	C39	D30	Oral	N4	16	No	+
CDC F003000402	A8	B34	C39	D30	Oral	N4	256	No	+
CDC F003000418	A19	B35	C40	D31	Oral	N4	0·125	No	+

(*Continued*)

Table 1. Cont.

Code no.	PFGE karyotype (excluding R chromosome)	PFGE *Sfi*I	PFGE *Bss*HII	Rep PCR	Source of isolation*	Geological origin (region†/hospital)	Fluconazol MIC ($\mu g\ ml^{-1}$)	Nosocomial infection	HIV status
CDC F003000419	A19	B35	C40	D31	Oral	N4	128	No	+
CDC F003000420	A19	B35	C40	D31	Oral	N4	32	No	+

ND, Not determined.

*CVP, central venous pressure line; PCN, percutaneous nephrostomy.

†N, north; S, south; M, middle; E, east.

resistance, DNA sequencing may be more suitable than the genome fingerprinting methods used in this study (Lee *et al.*, 2004). Methods based on the *C. albicans* nucleotide sequence (such as the MLST) as a robust characterization system and the possibility of storing data in a central database (http://calbicans.mlst.net) have been proposed. This greatly facilitates standardization and international data exchange of molecular typing information via the internet for global epidemiology (Dodgson *et al.*, 2003; Robles *et al.*, 2004; Tavanti *et al.*, 2004). The results from PFGE-RFLP approaches are often laboratory-dependent; however, with standardized protocols, inter-laboratory comparison of data is still feasible. Before sequence-based typing methods such as MLST gain global consensus and popularity, the highly discriminatory PFGE-RFLP approaches demonstrated here still remain useful and cost-effective tools in outbreak investigation.

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Mycology

Susceptibilities to amphotericin B and fluconazole of Candida species in TSARY 2002

TSARY Hospitals
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Abstract

Susceptibilities to amphotericin B and fluconazole of 909 *Candida* species collected during the Taiwan Surveillance of Antimicrobial Resistance of Yeasts (TSARY) in 2002 were determined by the broth microdilution method. There were 395 (43.5%) *Candida albicans*, 244 (26.8%) *C. tropicalis*, 187 (20.6%) *C. glabrata*, 63 (6.9%) *C. parapsilosis*, 9 (1%) *C. krusei*, and 11 (1.2%) others. Among them, 23 (2.5%) isolates were resistant to amphotericin B. They consisted of 10 *C. glabrata*, 6 *C. krusei*, 3 *C. albicans*, 1 *C. tropicalis*, 1 *C. parapsilosis*, and 2 others. The resistance rate to amphotericin B has increased compared with that of TSARY 1999 (2.5% versus 0.5%). There were 7 *C. krusei*, 5 *C. albicans*, 3 *C. glabrata*, and 2 others isolates resistant to fluconazole. The resistance rate to fluconazole has decreased from 8.4% in 1999 to 1.9% in 2002. A pattern of coresistance to both amphotericin B and fluconazole was observed.
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Keywords: *Candida*; Susceptibility; Resistance

1. Introduction

Nosocomial infections caused by yeasts have increased significantly in the past 2 decades. The prevalence of nosocomial candidemia increased 27-fold from 1981 through 1993 at a major hospital in Taiwan (Chen et al, 1997; Hung et al, 1996). In the United States, yeast infection also ranks as the fourth most common cause of nosocomial bloodstream infection (Beck-Sague and Jarvis, 1993; Pfaller et al, 1998). The dramatic increase in the prevalence of fungal infections is probably the result of alterations in immune status and invasive hospital procedures (White et al, 1998; Yang and Lo, 2001). Thus, infections caused by *Candida* species are becoming important causes of morbidity and mortality in immunocompromised patients. The major issues concerning currently available antifungal drugs include side effects and ineffectiveness against certain fungi. Because of broad prophylactic use and long-term treatment with antifungal drugs, drug resistance has become an important issue in various fungal infections, which have profound effects on human health (Marr et al, 2001; Pfaller et al, 2003; Yang et al, 2004b).

Candida species have various degrees of susceptibility to common antifungal drugs. For instance, *Candida lusitaniae* is relatively resistant to amphotericin B (Hadfield et al, 1987), whereas *C. krusei* and *C. glabrata* are less susceptible to fluconazole than other *Candida* species (Akova et al, 1991; Orozco et al, 1998; Yang et al, 2004b). This phenomenon emphasizes the importance of identification and surveillance of the *Candida* species in the clinical settings.

As part of the national survey Taiwan Surveillance of Antimicrobial Resistance of Yeasts (TSARY) in 1999, 22 hospitals have contributed 660 clinical yeast isolates to our study (Lo et al, 2001). Among the 632 tested isolates, 0.5% and 8.4% of isolates were resistant to amphotericin B and fluconazole, respectively. We have also shown that the levels of susceptibility to fluconazole of *Candida* species are different among different species (Yang et al, 2004a; Yang et al, 2004b). The aim of this study is to determine the susceptibilities to amphotericin B and fluconazole of isolates

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collected in a follow-up survey in 2002, TSARY 2002, and to investigate the change of susceptibility to antifungal drugs of *Candida* species in Taiwan from 1999 to 2002.

2. Materials and methods

2.1. Organisms and media

Yeast isolates were collected from 24 hospitals that participated in TSARY 2002. Each hospital was asked to submit all yeast pathogens from blood and up to 10 *C. albicans* and 40 non-*albicans Candida* species isolates from nonsterile sites from June to August in 2002. Only one isolate was accepted during each episode of infection. Isolates were stored frozen at −70°C in bead-containing Microbank cryovials (PRO-LAB Diagnostics, Austin, TX). The isolates were first subcultured on Sabouraud dextrose agar (BBL, Becton Dickinson Cockeysville, MD) to assess the purity and identification after they were sent to the laboratory at National Health Research Institutes (NHRI). Pure isolates were labeled and stored in vials containing 50% glycerol at −70°C awaiting further analysis.

2.2. Identification

The identifications of the isolates were reassured in the laboratory at the NHRI. The identification procedure for the yeast isolates was performed as previously described (Lo et al, 2001). Isolates identified as *C. albicans* by participating hospitals were first subjected to the germ tube test in brain heart infusion (BHI, BBL) medium containing 10% goat serum (GibcoBRL 16210-064, Grand Island, NY) at 37°C for 2–3 h (Larone, 1995). The VITEK Yeast Biochemical Card (bioMérieux, St. Louis, MO) was then used to identify the isolates appearing to be negative by the germ tube assay in the NHRI laboratory and the isolates were identified as non-*albicans Candida* species by participating hospitals. Api32C (bioMérieux) was used to assess the NHRI result when the VITEK Yeast Biochemical Card showed less than 90% confidence.

2.3. Antifungal susceptibility testing

The minimum inhibitory concentration (MIC) to amphotericin B and fluconazole of each yeast was determined by in vitro antifungal susceptibility testing according to the guidelines of M27-A published in 1997 by the National Committee for Clinical Laboratory Standards (1997). The RPMI medium 1640 (31800-022) provided by GibcoBRL was used for the testing. Strains from American Type Culture Collection were used as the standard controls. The final growth of each isolate was measured by a Spectra MAX Plus (Molecular Devices Corp, Sunnyvale, CA) after 48-h incubation at 35°C. We also measured MICs of some isolates by Etest (AB Biodisk Solna, Sweden) to assess the results of the broth microdilution method.

The interpretation of MICs was according to the guidelines of the National Committee for Clinical Laboratory Standards (1997). The MICs to amphotericin B and fluconazole were defined as the MICs of drugs capable of reducing the turbidity of cells to greater than 95% and 50%, respectively. Isolates with MIC ≥ 2 μg/mL were considered

Table 1
The susceptibility to fluconazole of *Candida* species from different sources

Susceptibility	*C. albicans*	*C. tropicalis*	*C. glabrata*	*C. parapsilosis*	*C. krusei*	Others	All
Urine							
S	98 (93.3)	107 (95.5)	71 (52.2)	8 (100)	0	0	284 (78.2)
SDD	5 (4.8)	5 (4.5)	63 (46.3)	0	0	0	73 (20.1)
R	2 (1.9)	0	2 (1.5)	0	2 (100)	0	6 (1.7)
Sputum							
S	112 (91.3)	56 (96.6)	5 (35.7)	1 (100)	0	2 (50)	176 (87.6)
SDD	6 (5)	2 (3.4)	8 (57.2)	0	1 (25)	1 (25)	18 (8.9)
R	2 (1.7)	0	1 (7.1)	0	3 (75)	1 (25)	7 (3.5)
Blood							
S	55 (98.2)	30 (96.8)	6 (46.2)	24 (100)	0	0	115 (91.3)
SDD	0	1 (3.2)	7 (53.8)	0	0	0	8 (6.3)
R	1 (1.8)	0	0	0	1 (100)	1 (100)	3 (2.4)
Wound							
S	21 (95.5)	9 (100)	0	12 (100)	0	2 (100)	44 (95.7)
SDD	1 (4.5)	0	1 (100)	0	0	0	2 (4.3)
R	0	0	0	0	0	0	0
Others							
S	87 (94.6)	33 (97.1)	11 (47.8)	18 (100)	1 (50)	3 (75)	153 (88.4)
SDD	5 (5.4)	1 (2.9)	12 (52.2)	0	0	1 (25)	19 (11)
R	0	0	0	0	1 (50)	0	1 (0.6)
All							
S	373 (94.4)	235 (96.3)	93 (49.7)	63 (100)	1 (11.1)	7 (63.6)	772 (84.9)
SDD	17 (4.3)	9 (3.7)	91 (48.7)	0	1 (11.1)	2 (18.2)	120 (13.2)
R	5 (1.3)	0	3 (1.6)	0	7 (77.8)	2 (18.2)	17 (1.9)

S = susceptible; SDD = susceptible and dose-dependent; R = resistant. Data are given as number of isolates (%).

Table 2
The susceptibility to amphotericin B of *Candida* species

MIC (μg/mL)	*C. albicans*	*C. tropicalis*	*C. glabrata*	*C. parapsilosis*	*C. krusei*	Others	Total
0.06	3 (0.8)	1 (0.4)	0	0	0	0	4 (0.4)
0.125	1 (0.2)	0	0	0	0	0	1 (0.1)
0.25	6 (1.5)	1 (0.4)	1 (0.5)	6 (9.5)	0	1 (9.1)	15 (1.7)
0.5	218 (55.2)	126 (51.7)	33 (17.6)	27 (42.9)	1 (11.1)	4 (36.4)	409 (45)
1	164 (41.5)	115 (47.1)	143 (76.5)	29 (46)	2 (22.2)	4 (36.4)	457 (50.3)
2	3 (0.8)	1 (0.4)	10 (5.4)	1 (1.6)	6 (66.7)	2 (18.1)	23 (2.5)
Total	395	244	187	63	9	11	909
MIC_{50}	0.5	0.5	1	0.5	2	1	1
MIC_{90}	1	1	1	1	2	2	1

Data are given as number of isolates (%).

resistant to amphotericin B. Isolates with MIC ≤ 1 μg/mL were considered susceptible. Isolates with MIC ≥ 64 μg/mL were considered resistant to fluconazole, whereas isolates with MIC ≤ 8 μg/mL were considered to susceptible. Isolates of which the MICs fell in between (16–32 μg/mL) were fluconazole-susceptible/dose-dependent. MIC_{50} and MIC_{90} were defined as the MICs of 50% and 90% of the total population.

2.4. Database and analysis

The database for this study contained the characteristic information of each submitted isolate: hospital origin, location and type of the hospital, and identification and source of the isolate. The statistical significance of the differences in frequencies and proportions was determined by the χ^2 test with Yates' correction.

3. Results and discussion

3.1. Susceptibilities to fluconazole of Candida species from different sources

A total of 909 isolates listed in Table 1 were analyzed for their susceptibilities to amphotericin B and fluconazole. *C. albicans* was the most common species among the isolates (43.5%). *C. tropicalis* (26.8%) and *C. glabrata* (20.6%) were 2 most common non-*albicans Candida* species followed by *C. parapsilosis* (6.9%), *C. krusei* (1%), and others (1.2%). When classified according to the sources, there were 363 (39.9%) isolates from urine, 201 (22.1%) from sputum, 126 (13.9%) from blood, 48 (5.3%) from central venous line, 46 (5.1%) from wound, 33 (3.6%) from ascites, 14 (1.5%) from pus, and 78 (8.6%) from other sources.

A total of 772 (84.9%), 120 (13.2%), and 17 (1.9%) isolates were fluconazole-susceptible, fluconazole-susceptible/dose-dependent, and fluconazole-resistant, respectively. The MIC_{50} and MIC_{90} of these isolates were 1 and 16 μg/mL, respectively. The 17 fluconazole-resistant isolates consisted of 7 from sputum, 6 from urine, 3 from blood, and 1 from perineum. Fewer isolates (1.9%) from TSARY 2002 were resistant to fluconazole than that in TSARY 1999 (8.4%, $P < 0.05$) (Yang et al, 2004b). In contrast, higher percentage of isolates from TSARY 2002 (13.2%) were susceptible-dose dependent than that in TSARY 1999 (7.1%, $P < 0.05$). Consequently, there were similar portions of isolates susceptible to fluconazole in both surveys. The fluconazole resistance rate of isolates from blood in TSARY 2002 was 2.4%, which is higher than what has been reported from one major hospital in Taiwan (1.3%) (Chen et al, 1996). The MIC_{50} of *C. krusei* was 64 μg/mL. *C. krusei* (77.8%) had the highest resistance rate to fluconazole than any other species studied, which is consistent with previous report (Akova et al, 1991; Yang et al, 2004b). Although only 1.6% of *C. glabrata* were resistant to fluconazole, less than half of the isolates (49.7%) from this species were susceptible to fluconazole. The MIC_{50} and MIC_{90} of it were 16 and 32 μg/mL, respectively. In contrast, all of the *C. parapsilosis* isolates were susceptible to fluconazole, which is consistent with the previous report that *C. parapsilosis* is the most susceptible species to fluconazole (Yang et al, 2004b).

Table 3
The coresistance to amphotericin B and fluconazole

MIC of amphotericin B	MIC of fluconazole										Total
	0.125	0.25	0.5	1	2	4	8	16	32	64	
0.06		1	1	1		1					4
0.125	1										1
0.25		4	2	5	1	1		1		1	15
0.5	27	139	75	57	40	25	17	14	10	5	409
1	26	87	51	50	45	44	59	59	31	5	457
2		2	2	1	1	4	2	3	2	6	25
Total	54	233	131	114	87	75	78	77	43	17	909

3.2. Susceptibilities to amphotericin B of Candida species

The range of MICs to amphotericin B was from 0.06 to 2 μg/mL (Table 2). *C. krusei* was less susceptible to amphotericin B than any other species because the MIC_{50} of this species was 2 μg/mL. A total of 23 (2.5%) isolates were resistant to amphotericin B. Fungal infections caused by non-*albicans Candida* species have increased dramatically (Abi-Said et al, 1997; Slavin et al, 1995; Walsh et al, 2004), which was also reflected in the distribution of resistant isolates (Slavin et al, 1995; Walsh et al, 1998). Of the 23 amphotericin B-resistant isolates, 20 isolates were non-*albicans Candida* species. The distributions were 10 *C. glabrata*, 6 *C. krusei*, 1 *C. tropicalis*, 1 *C. parapsilosis*, and 2 others. Higher percentage of isolates (2.5%) from TSARY 2002 were resistant to amphotericin B than that in TSARY 1999 (0.5%, $P < 0.05$) (Yang et al, 2004b).

3.3. Coresistance to both amphotericin B and fluconazole

The phenomenon of coresistance has been reported for many pathogens. The trend of coresistance to amphotericin B and fluconazole is shown in Table 3. A total of 1.6% (12/772) of fluconazole-susceptible, 4.2% (5/120) of fluconazole-susceptible/dose-dependent, and 35.3% (6/17) of fluconazole-resistant isolates were resistant to amphotericin B. A total of 1.2% (11/886) and 26.1% (6/23) of isolates with MICs to amphotericin B of ≤1 and 2 μg/mL, respectively, were resistant to fluconazole.

A total of 11.1% of *C. krusei* and 49.7% of *C. glabrata* were susceptible to fluconazole, which is consistent with the previous report that both species were less susceptible to fluconazole than other *Candida* species (Akova et al, 1991; Orozco et al, 1998; Yang et al, 2004b). Thus, fluconazole is not a drug recommended to treat infections caused by these 2 species. Amphotericin B appears to be the drug of choice for the treatments. However, along with increased usage of amphotericin B, more *C. krusei* were resistant to it in TSARY 2002 (66.7%) than in TSARY 1999 (10%). This is also the case for *C. glabrata* (5.4% versus 0%). The coresistance to both amphotericin B and fluconazole of *C. krusei* and *C. glabrata* may become an issue for treatment of infections caused by them.

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新型恆溫式圈環形核酸增幅法簡介與應用
Loop-mediated Isothermal Amplification （LAMP）

前　言

核酸增幅技術已成爲生物學領域最重要的工具之一，在臨床醫學應用領域上，舉凡感染病原、遺傳疾病…等的檢測，皆扮演著舉足輕重的角色。除了一般人較熟知常用的 PCR 相關技術外，其他核酸增幅技術如依賴核酸序列擴增法（Nucleic acid sequence-based amplification, NASBA）、自主序列複製系統（self-sustained sequence replication, 3SR）、鏈替代擴增法 （strand displacement amplification, SDA）亦陸續被發展出來。這些核酸增幅技術，固然均具有快速且專一地增幅少量核酸片段之優點，但缺點則是：除 SDA 是在恆溫反應外，其餘均需要特殊且高精確度的快速溫度循環儀或檢測機器，易有僞陽性，核酸的濃度及純度要求高，且增幅效率不好。

2000 年日本 Notomi 等人研發出 LAMP 核酸增幅技術[1]，由於操作十分簡單，儀器僅需溫度可達 60-65℃恆溫的水浴槽或乾熱板（heat block），因此適合田間篩檢、現場 （point-of-care）或床邊（bed-side）測試、初級照護機構、小規模臨床實驗室、偏遠物資缺乏地區，田野分子流行病學調查或大規模檢疫工作用。

原　理

「LAMP」[1]全名爲 loop-mediated isothermal amplification，以名稱來看即一種恆溫、具 loop 形式的 DNA 擴增法。LAMP 反應最大的特點在於：（1）使用作用溫度 60-65℃和具高 DNA strand 置換能力的 DNA 聚合酶（DNA polymerase）：換個角度來說，就是缺乏 5’→3’的 DNA 分解能力，在 DNA 聚合的過程中，只能將模版 DNA 上既有的互補 DNA 鏈整條〝鏟〞起，就像是靠聚合酶進行 dsDNA→ssDNA 的步驟。（2）使用 inner primers（FIP, BIP）和 outer primers（F3, B3）（圖一），或再加上 loop primers（Loop F, Loop B）[2]：inner primers 與 outer primers 爲反應所必須，在增幅的過程中分別扮演了啓始引發與後續「self-priming」的角色，而 loop primers 則是互補於 DNA 序列 F1、F2 中間的片段，這個位置在 LAMP 的 DNA product 上，爲 stem-loop 結構的 loop 位置，有助於提升整個反應的效率；這三對引子在設計之時，T_m 值要控制在 60-65℃，以利 DNA 聚合時可達最佳效能，

也因此提升了在溫度調控上的便利性及專一性。除了以上的優點，目前的研究皆肯定 LAMP 的靈敏度可達 10 copies 以下[1]，再加上作用時間短且可以肉眼直視結果[3]，實爲一極佳的核酸檢驗技術。

LAMP 的反應原理，簡單來講，就如同一般的 PCR，但是其 denature 的步驟則由一特定的 DNA polymerase 取代，primers 的 T_m 值也全部控制在 60-65℃，而省去 annealing 的步驟，再加上其設計會使被擴增的 DNA 產物呈現 stem-loop 的結構，促使 self-prime 的發生而 DNA 片段長度持續以對數延長，過程中也不斷釋出啓始或中間反應的 DNA 片段，造成 target DNA 的質量與數量快速的放大，此結果有助於檢驗；整個反應過程，除了一開始需要一次 heat denature 和即時冰上冷卻的動作外，之後只要視需要設定 60-65℃之間的反應溫度，作用 35-60 分鐘，終止反應後，可以 magnesium pyrophosphate 產生沈澱的方式[4]，或利用 SYBR Green Ⅰ螢光顯色[3]來判斷檢體的陰、陽性。（詳細反應過程請詳閱 [1, 2]）

材料與方法

一、DNA polymerase

依據原理所敘述的功能，文獻[1]指出可選用下列三種 DNA polymerase 之一：*Bst* DNA polymerase, *Bca*BEST DNA polymerase, *Z-Taq* DNA polymerase。其中 *Z-Taq* DNA polymerase 的效果較差，但如果 DNA polymerase 必須在 heat denaturation 之前加入，*Z-Taq* DNA polymerase 則是很好的選擇。

二、Primers

在前面「原理」的地方有提到以下三組 primers：Outer primers、Inner primers 和 Loop primers。

在 DNA 片段上 primers 序列的位置，5’→3’分別是 B3-B2-Loop B-B1 和 F3-F2-Loop F-F1（圖一）。在 LAMP 進行的時候，因爲 outer primers（F 3, B 3）在 inner primers 的 5’端，所以會將下游 inner primers 所合成的 DNA 鏈鑅起。inner primers 則包括 forward inner primer（FIP）和 backward inner primer（BIP）。FIP, BIP 的設計則較特殊，從 5’→3’的序列組成爲 F1c-F2、B1c-B2 或 F1c-[Spacer]-F2、B1c-[Spacer]-B2，[Spacer]爲 3～6bp 的 T（thymidine）或不互補的序列。當 outer primers 啓始的 DNA 合成，使 inner primers 啓始的 DNA 鏈成爲單股時，inner primers 上的 F1c、B1c 會與其下游的 F1、B1 形成 looped out 的結構，最後由兩端 loop 的啞鈴形 DNA 衍生爲主要增幅步驟的 stem-loop 模版 DNA，loop 端的單股 DNA 序列 5’→3’爲[Spacer]-F2-Loop F、[Spacer]-B2-Loop B 或 F2-Loop F、B2-Loop B（圖二）。

Loop primers（Loop F、Loop B）的功能則是在 stem-loop 模版 DNA 增幅過程中，當 loop 端的序列 5’→3’爲[Spacer]c-F2c-Loop Fc、[Spacer]c-B2c-Loop Bc 或 F2c-Loop Fc、B2c-Loop Bc 時，加強增幅效率[2]。

三、Reaction mixture for LAMP

根據原創文獻[1]與之後的引用文獻記載，總 LAMP reaction mixture 爲 25 μl，除了 dNTP（0.4-1.6mM each dNTP）, primers, template DNA 與 $MgSO_4$ 外，以下爲較固定成分：1 M betaine（Sigma）, 20 mM Tris-HCl（pH 8.8）, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100。

$MgSO_4$ 濃度的調整，是由於結果的分析是以 magnesium pyrophosphate formation 產生沈澱的方式，而有所不同[4]。

LAMP reaction mixture 加入了 outer primers（濃度要低於 inner primers）, Inner primers, 或 Loop primers[2]與 template DNA 加入後，加熱至 95℃ 5 分鐘再置冰上冷卻，加入 8 U *Bst* polymerase，置於 65℃反應 35 分鐘至 1 小時，80℃ 10 分鐘結束反應後，就可進行結果的判讀[1, 2]。反應過程中的 heat denaturation 及 DNA polymerase heat termination，有文獻[5]指出可省略而不影響最後結果，雖然反應的啓始較慢，但對檢驗器材的精簡有很大的幫助。

四、Analysis

就較直接結果判讀來說，magnesium pyrophosphate 沈澱[4]所造成的渾濁度可以肉眼或濁度計 405nm 檢測[6]，也可以快速離心，以得到更確切的結果。再者在反應結束後加入高濃度的 SYBR Green I 螢光顯色直接以肉眼判斷[3]。

檢驗應用

LAMP 自推出以來有關應用可行性的報告不斷在擴增中。在病原菌檢測上用於直接檢測病患痰液中的 *Mycobacterium tuberculosis* Complex, *M. avium* 及 *M. intracellulare*[3]及牛隻的 *M. avium* subsp. *paratuberculosis*。亦有學者發展 Real-time LAMP 可即時追蹤濁度變化以偵測西尼羅河病毒（West-Nile Virus）[7]，並展望此技術未來除檢測病人檢體外亦可應用於田野監測野鳥及病媒蚊帶病毒情形。LAMP 可應用於偵測人類血漿中的皰疹病毒-6 [8]。亦有學者描述 LAMP 技術應用於原位（*in situ*）偵測 *Escherichia coli* O157:H7 的 stx_2 基因[5]。使用簡便的 LAMP 技術偵測非洲昏睡病的錐體蟲（trypanosomes），可有利於在開發中國家推廣[9]。在植物檢疫上，在日本 LAMP 被應用來篩檢日本蕃薯苗圃感染嵌紋病毒[6]及蕃茄植株和白蠅病媒體內的黃葉捲曲病毒[10]。在漁業養殖方面，LAMP 可檢測蝦子白斑病病毒 [11]及魚的 *Edwardsiella tarda* 感染[12]。LAMP 的產物爲標的 DNA 反轉重複（inverted repeats）的啞鈴形 DNAs 形成帶有複環形狀的花椰菜狀構造。此產物可用 *Tsp*RI 等限制酵素切後再以引子延長反應，取得大量的單股 DNA 產物，作爲後續定序確認或 DNA 微陣列的材料[13]。最近則有香港地區報告 SARS 檢驗之使用[14]。

優點

一、操作簡便，步驟簡單，所有反應在單一試管內完成，增幅 DNA 在 60-65℃恆溫下進行即可，DNA 可以不變性爲單股，不需經繁複的核酸萃取可直接從檢體檢測，不需要溫度循環器、跑膠或雜合偵測等複雜的設備，就可以完成。

二、特異性相當高，因爲需兩對引子先後辨認六段序列，反應才能持續進行。

三、高效率，每次循環可增幅三倍，且不需 PCR 的階段性循環升降溫，故所需時間較 PCR 短（一般約 35 分-1 小時），只要數個 copy 的標的基因即會快速增幅至 10^9 個 copy（500 μg/ml DNA），所需樣本量微，敏感度與 nested- 或 real-time PCR 相當，且檢測容易。

四、分析簡便，DNA 大量增幅過程中，會有大量副產物 pyrophosphate 產生，藉由 magnesium pyrophosphate 白色沈澱物的生成，可肉眼辨識混濁度或以簡單濁度計量測，且沈澱物量與 DNA 產物成正比。若加入 SYBR Green I 螢光染劑，目測之靈敏度可與電泳凝膠法相當。

五、也可檢測 RNA（RT-LAMP），只要在同一反應管中多加反轉錄酶（reverse transcriptase），先行

反轉錄的過程，再進行 LAMP 即可。

六、可偵測 SNP，因爲每次循環只要有單一核甘酸變異即會被偵測出來。

缺點

一、primer 設計複雜且限制多，目前網站上已有專用的設計程式，惟尚未對外開放。

二、標準化不易。

三、迄今發表之應用尚少，關於其侷限性仍有待釐清。

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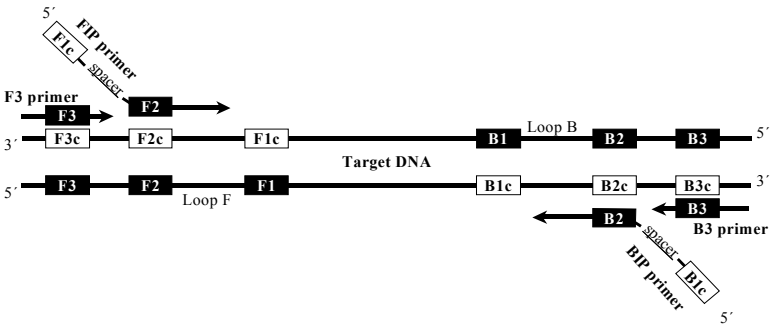
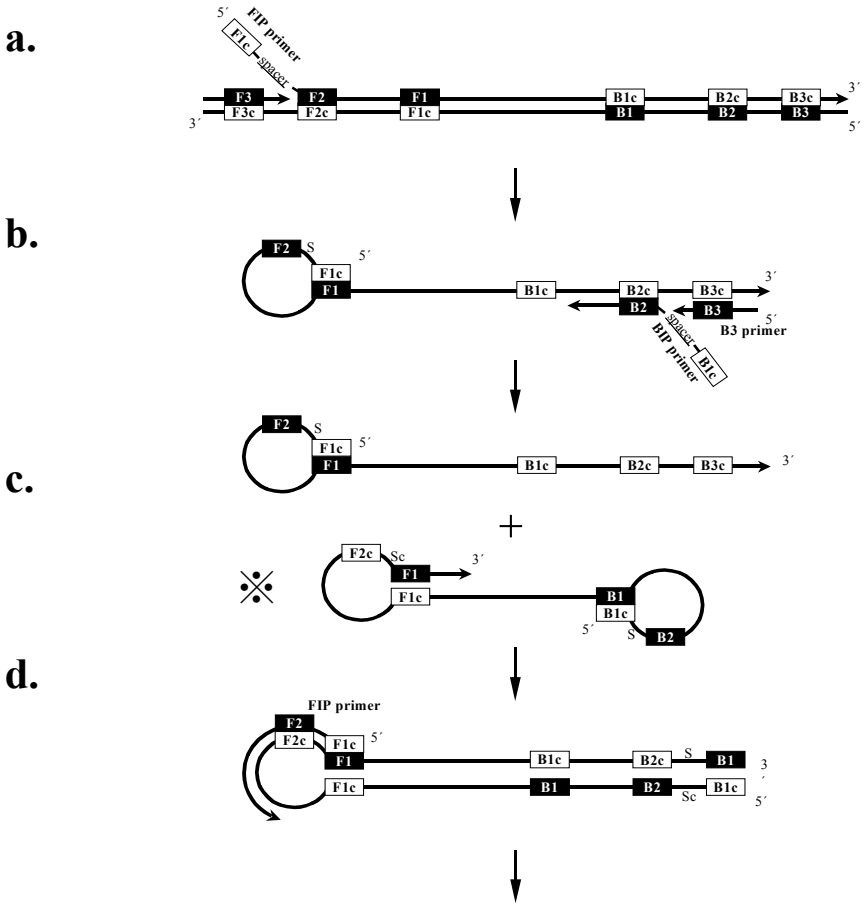
圖一、LAMP 的 primer 與標的 DNA 關係圖（改編自[11]Fig.1A）



FIP & BIP：inner primer　　F3 & B3：outer primer

Inner primer 相對於 outer primer 在 DNA 片段上的結合位置，是在其下游。由於 LAMP 所選用特定 DNA 聚合酶的功能，致使在 outer primer 下游的 inner primer 啓始合成的 DNA 鏈，在 outer primer 進行 DNA 聚合的過程中被鑲起。而 inner primer 上 F1c & B1c 的序列，在 LAMP 的反應過程中，將形成主要的 LAMP 啞鈴形模版 DNA（見圖二 c）。

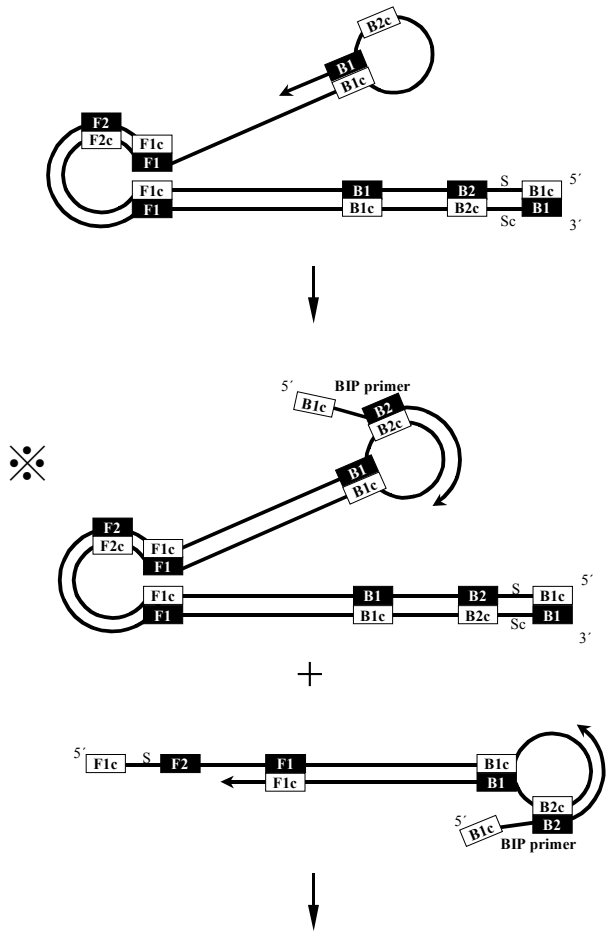
圖二、LAMP 簡略流程圖（改編自[1]Fig.1）



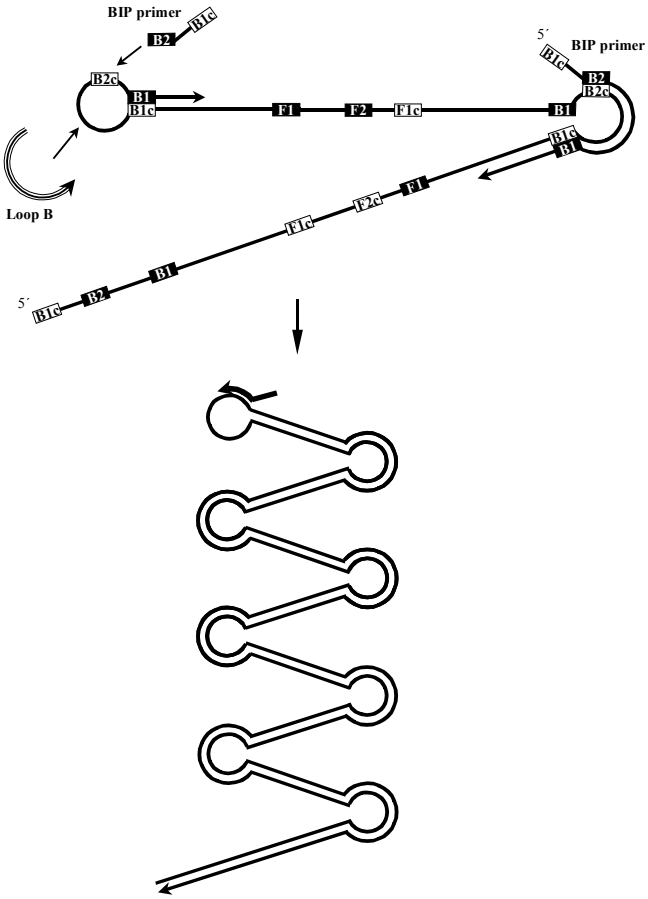
a.～ c.

以 FIP 啓始的 DNA 合成爲例（a），因上游自 F3 合成 DNA 的同時，特定 DNA 聚合酶作用使 FIP 啓始合成的 DNA 鏈脫離原始模版 DNA；同理另一端 B3 與 BIP 的 DNA 合成後（b），產生一特殊 DNA 片段，因兩端具有與較中間特定區域（F1、B1）互補的序列（F1c、B1c），而形成「啞鈴形」（兩端圈環）的結構（c※）。圈環（loop）的形成則造就 self-primed 的功能。

e.



f.



g.

d.～ g.

構成 stem-loop 的 DNA，在圈環的部分可再與 inner primer 接合，繼續 DNA 的合成（d），再加以 self-primed 致使 stem-loop DNA 會自行延伸（e-f），且過程中製造出的多重產物，部分再繼續回復循環於整個 LAMP 過程中，如此持續循環作用，加速標的 DNA 的產能不斷增殖，而利於檢測應用。

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