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病原真菌快速檢驗及分子流行病學群體計畫 III

利用 Real-Time Light-Cycler PCR 技術快速鑑定高危險群患者血液中的病原真菌-III

研 究 報 告

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中文摘要

侵襲性黴菌感染日益增加，診斷困難，且伴隨高死亡率。隨著新的較安全、較昂貴的抗黴菌藥物引起，正確且快速的診斷方法的重要性更是迫切。我們之前的研究建立以 Real-Time Light-Cycler PCR 及偵測系統可偵測 6 種 yeast 的 DNA，增幅 ITS1 及 ITS2 之片斷，敏感性及特異性很高。為了應用到臨床檢體，本研究建立由血液檢體中抽取黴菌 DNA 的技術，以全血加入不等量之 yeast，發現此方法之敏感性高達 1cfu/ml 之全血。應用於 58 個血液培養陽性之檢體，由血瓶中抽取純化之 DNA，此方法可 100%正確偵測及鑑定菌種。要應用於臨床檢體直間偵測黴菌有若干課題需克服。總之，以 real-time PCR 放大增幅黴菌 ITS1 及 ITS2 之 DNA 片斷，是個有潛力的快速診斷方法。

關鍵詞：real-time Light-Cycler PCR assay, **念珠菌**

英文摘要

The increasing incidence of invasive fungal infections which are associated with significant morbidity and mortality in immunocompromised patients and critically ill patients and the recent availability of expensive antifungal agents with better safety profiles, emphasized the need to improve the currently limited diagnostic tools. We have established the Light-Cycler PCR and detection system (Roche Diagnostics, Mannheim, Germany) that specifically amplified a DNA fragment between inter-spacer 1 (ITS1) region and ITS2 region. Methods for extraction of DNA of fungi from whole blood were established which was designed to isolate DNA from yeasts and molds and to avoid contamination from common reagents. Using whole blood spiked with serial dilution of yeast suspension, the sensitivity of this assay was determined. Up to one colony-forming unit of *Candida* per mL of whole blood was detectable. A total of 58 positive blood cultures containing yeasts were analyzed by the real-time PCR for species identification. From these blood cultures, 59 strains of yeasts were isolated, which included *C. albicans* (17 strains) , *C. tropicalis* (10 strains), *C. glabrata* (8 strains), *C. parapsilosis* (7 strains), *C. neoformans* (7 strains), and *C. krusei* (10 strains). All strains of the above species were correctly identified, resulting in a test sensitivity of 100% for each of the above seven species. Coexisting bacteria in blood specimens did not produce any detectable PCR products and did not interfere with yeast identification. To apply this PCR assay in the clinical practice, several issues were important and discussed. In conclusion, the real-time Light-Cycler PCR assay combines rapid *in vitro* amplification of DNA with

real-time species determination and quantification. This method is simple, rapid, sensitive, and cost-effective and seems to be very promising.

Key words: real-time Light-Cycler PCR assay, *Candida*

(1)前言

Invasive fungal infection has been increased in the past decade and carried significantly high mortality and morbidity in immunocompromised patients and critically ill patients [1-7]. However, diagnosis of invasive fungal infection, particularly chronic invasive candidiasis, invasive aspergillosis and invasive zygomycosis, is notoriously difficult [8]. Therefore, the epidemiology of invasive fungal infection other than candidemia or certain rare fungi is lacking in Taiwan despite of increasing empirical use of systemic antifungal therapy. Treating this infection as early as possible is one of the prerequisites for a favorable outcome [1], but since no reliable means of early diagnosis exists, it has become commonplace to treat patients at high risk empirically with amphotericin B when there is clinical suspicion of invasive fungal infection [9]. Therefore, alternative strategies are needed for the early recognition of invasive fungal infection to allow better selection of patients who need treatment, while reducing the number of patients who are exposed unnecessarily to conventional amphotericin B which has significant adverse effects [10]. In addition, better selection of patients who require treatment for invasive fungal infection will also help to contain the cost of treatment when novel and expensive drugs are used [11, 12]. Furthermore, increasing use of fluconazole and amphotericin B raise the concern of emergence of resistance and alternative antifungal agent is limited, if any, at present time [13].

To address this issue, an alternative approach is warranted. A rapid and sensitive diagnostic method, such as polymerase chain reaction (PCR) is considered. However, establishing a timely and clinically useful PCR assay

for invasive fungal infection has faced several aspects of difficulties. First, it must involve simple, efficient sample preparation directly from blood; this may include a means of concentrating low numbers of colony forming units in a blood sample [14, 15]. Second, as fungal cell wall is complex, protocols for extraction of DNA of fungal cells either are vary time-consuming or show poor release of fungal DNA compared to methods of extraction of DNA of human cells or viruses [15]. Third, extreme care must be taken to avoid false-positive or false-negative results. False-negative results can be monitored by the use of competitive PCR [16]. However, false-positive results are more difficult to control. Since conidia are often present in the air, false-positive results can be generated by the transient presence of *Aspergillus* in the respiratory tract [17].

(2)材料與方法

Fungal strains. Sevene reference strains including *Candida albicans, Candida glabrata, Candida guilliermondii, Candida krusei, Candida parapsilosis, Candida tropicalis*, and *Cryptococcus neoformans* were used in this study.

Preparation of template DNA from *Candida* blastoconidia suspended in blood. In order to determined the sensitivity of the PCR assay, blood samples from healthy volunteers was seeded with known numbers of *Candida* blastoconidia. Fungal isolates were cultures on *Sabouraud medium* at 30°C; *Candida* isolates were cultured for 48 h, and *Cryptococcus* isolates were cultured for 72 h. Thereafter, fungal saline suspensions were adjusted photometrically (A_{530}; McFarland no. 0.5 standard) to a concentration of $1x10^6$ cells/mL. Tenfold serial dilution of fungal suspensions (10^6 to 10^0 cells) was spiked into 5 mL of whole blood to test the sensitivity and specificity of the assays.

Fungal DNA was extracted from 5 mL of whole blood in a biosafety hood in a separate room provided with equipment exclusively used for DNA extraction. Whole blood was collected in EDTA to reduce DNA degradation and stored at 4°C before processing. Leukocytes and fungal cells floating in the blood or found within human leukocytes (buffy coat layer) were separated by centrifugation at 800 x g for 10 min at room temperature. RBC lysis solution (10 mM Tris [pH7.6], 5 mM MgCl2, 10 mM NaCl) was added to the buffy coat, inverted to mix and was incubated at room temperature for 10 min. The buffy-coat layer was further incubated with leukocyte lysis buffer (10

mM Tris [pH 7.6], 10 mM EDTA [pH8.0], 50 mM NaCl, 0.2% sodium dodecyl sulfate, 200 μg of proteinase K [Boehringer Mannheim] per mL) at 65°C for 45 min. Then, the samples were centrifuged at 14,000 rpm for 20 min and incubated with 0.2 mL of 50 mM NaOH at 95°C for 10 min. The pellet was neutralized with 1 M Tris (pH 7.0) and further treated with 300 μg of Zymolyase (ICN, Costa Mesa, Calif.) per mL in 50 mM Tris (pH 7.5), 10 mM EDTA, and 0.2% β–mercaptoethanol at 37°C for 45 min to give spheroplasts. After centrifugation at 5,000 x *g* the supernatant containing human DNA and proteins was decanted. The pellet was resuspended in 1 M Tris-EDTA and was treated with 25 μl of 10% SDS. The sample was inverted to mix and incubated at 65°C for 30 min for protoplast lysis. Then, 0.4 mL of 5M potassium acetate was added and the sample was inverted to mix and was incubated at –20°C for 30 min for protein precipitation. After centrifugation at 12,000 x *g* for 20 min, DNA precipitation from the supernatant was carried out at room temperature for 5 min with isopropanol. DNA was purified with 70% ethanol, precipitated with cold centrifugation at 12,000 x g for 15 min, air dried, and resuspended in 20 ul of H_2O. Half of the eluent was used for amplification.

Clinical specimens. Blood samples were collected from the National Cheng Kung University Medical Center, Tainan, Taiwan, and from Chang Gung Memorial Hospital. BACTEC blood culture bottles (Becton Dickinson Microbiology Systems, Cockeysville, Md.) were normally inoculated with 3 to 10 ml of blood from patients, inserted into the BACTEC NR660 instrument (Becton Dickinson Microbiology Systems), and incubated at 37°C. Gram stain smears of aliquots from positive bottles were prepared to check for

the presence of **yeast**s. A total of 58 positive blood culture bottles containing **yeast**s were analyzed in this study. The blood **yeast**s isolated on subculture plates were identified by conventional procedures based on phenotypic and biochemical reactions (18).

Isolation of yeast DNA from positive blood cultures. The method of Fujita et al. (19) was used with a small modification to extract **yeast** DNA from the positive culture broths. (20) An aliquot (0.2 ml) of positive broth containing **yeast**s was added to 0.8 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) containing 0.05% proteinase K (Worthington Biochemical Inc., Lakewood, N.J.) and 0.05% Tween 20. The cell suspension was incubated at 55°C for 30 min and then centrifuged at 8,000 × *g* for 10 min in a microcentrifuge. The pellet was washed with 0.5 ml of TE buffer containing 0.5% Tween 20 and then with 0.5 ml of SE solution (1 M sorbitol, 0.1 M EDTA). After centrifugation at 8,000 × *g* for 10 min, the pellet was suspended in 0.5 ml of Lyticase solution (10 mg/ml; Sigma Chemical Co., St. Louis, Mo.) and incubated at 37°C for 1 h. After centrifugation, the pellet was suspended in 10 μl of TE buffer, and 1 μl of the suspension was added to 19 μl of microLYSIS solution. The suspension was heated in a thermal cycler to extract **yeast** DNA. (Seven randomly selected positive blood cultures containing bacteria were processed in the same manner for DNA extraction.) In addition, DNA was extracted from two blood samples from healthy individuals for PCR assay.

Light-Cycler-based PCR. The Light-Cycler PCR and detection system (Roche Diagnostics, Mannheim, Germany) was used for amplification and

quantification. PCR was performed in glass capillaries and cycling achieved by alternating heated air and air of ambient temperature, which ensures rapid equilibration between the air and the reaction components due to the high surface-to-volume ratio of the capillaries. Universal primers ITS1-4 and species-specific primers (CALB, CGL, CPA, and CTR) designed between ITS1 and ITS2 regions [21, 22] were used, as described previously [23]. For amplicon detection, the Light Cycler FastStart DNA Master SYBR Green Kit was used as described by the manufacturer. The PCR mixture (20µl) contained *Taq* polymerase, 1× Light Cycler reaction buffer, 3mM magnesium chloride, 0.5µM of primers. Template DNA was added at a final concentration of 1ng per 20µl of reaction mix. Samples were run in parallel by performing 35 cycles of repeated denaturation (5s at 95° C), annealing (5s at 58° C), and enzymatic chain extension (25s at 72° C). This step was followed by a melting curve analysis from 60 to 95° C and afterwards cooling to 40° C. The PCR run was completed within 45 min. The PCR process was then monitored by fluorescence quantification of the DNA-binding dye SYBR Green 1 dye for the detection of double-stranded DNA.

Comtamination precautions. The measures used to avoid PCR assay contamination included the use of separate rooms and glassware supplies for PCR setup and products, aliquoted reagents, positive-displacement pipettes, aerosol-resistant tips, multiple negative controls and low-copy-number positive controls [17] and as described by Fujita et al. [18]. Appropriate negative controls were included in each test run, including controls omitting either the primer or the DNA template during PCR assays. Before any stage of the experiment (DNA extraction, PCR master-mix preparation, addition of

template to master-mix, PCR amplification) was performed, all work-surfaces including cabinets, pipettes, racks and microcentrifuges, including rotors and adaptors, were wiped down with Microsol (Anachem) and DNAzap (Ambion) [46].

Determination of sensitivity of PCR for blood samples.

Agarose gel electrophoresis. Gel electrophoresis was conducted in TBE buffer (0.1 M Tris, 0.09 M boric acid, 1 mM EDTA [pH 8.4]) at 100 V/cm for 1 to 2 hours by using gels composed of 2.0% (wt/vol) agarose (BioWhittaker Molecular Applications: BMA, Rockland, ME, USA) and stained in 0.5 µg/ml ethidium bromide solution for 15 min, followed by washing for 30 min each with distilled H_2O.

(3)結果

Specificity of Light-Cycler amplification. The *C. albicans* specific primers CALB1 and CALB2 were annealed with DNA extracted from whole blood spiked with *C. albicans ATCC14053*. No cross-reaction with DNA from other fungal species was observed (data not shown). The detection limit was 5 cfu per PCR, i.e., 1 cfu/mL of blood (Fig 1).

Sensitivity of assays. DNAs of six *Candida* species and one *Cryptococcus neoformans* were successfully amplified from whole blood by their respective primers with the Light-Cycler and the detection limit of this method was 1 cfu per mL of whole blood (Fig 3). On the other hand, the detection limit of yeasts in blood cultures was 10 cfu per mL.

Identification of yeasts in positive blood cultures. A total of 58 positive blood cultures containing yeast**s** were analyzed by the real-time PCR for species identification. From these blood cultures, 59 strains of yeasts were isolated, which included *C. albicans* (17 strains) , *C. tropicalis* (10 strains), *C. glabrata* (8 strains), *C. parapsilosis* (7 strains), *C. neoformans* (7 strains), and *C. krusei* (10 strains). All strains of the above species were correctly identified, resulting in a test sensitivity of 100% for each of the above seven species. Coexisting bacteria in blood specimens did not produce any detectable PCR products and did not interfere with yeast identification.

(4)討論

. The increasing frequency of invasive fungal infection and the high mortality associated with disseminated fungal disease have underscored the importance of rapid detection of pathogenic fungi. Thus, rapid and correct identification to the species level is crucial for better clinical management of patients, as certain species are associated with higher mortality and increased virulence and vary in their resistance to antifungal therapy. Traditional identification methods based on phenotypic features are sometimes time-consuming and depend largely on the expertise of technicians. Therefore, we evaluated the feasibility of real-time LightCycler PCR amplification of the rDNA region, followed by melting curve analysis for the identification of clinically important yeasts. Besides, the sensitivity of the real-time PCR provides the potential for direct identification of yeast from blood samples.

However, this study demonstrated that in order to apply fungal PCR assays into the clinical practice, several issues should be carefully addressed.

1. Amount of clinical samples. It has been suggested that the fungal load in blood samples is frequently lower than 10 cfu/mL of blood, with 25% of cases having a fungal load of 1 cfu/mL or below. Thus, 5 mL of whole blood was collected to extract fungal DNA.
2. Methods for extraction of DNA of fungal pathogens from blood samples. The sensitivity of PCR assays is hampered by the low colony count of fungi in the clinical specimen, including blood [14,16], and the difficulty in releasing fungal DNA in the preparation of clinical specimen due to the

cell wall structure of the fungi. Furthermore, steps should be included to removal of the PCR inhibitors in the blood or blood cultures [24].

3. Contaminations occurring in fungal PCR assays. Contamination has been the main obstacle to the clinical application of PCR, especially in amplification techniques based on the use of pan-fungal primers. As yeasts and molds are ubiquitous in the environment, controlling contamination of extraction and PCR reagents is essential. Contaminations occurred during blood sampling as well as processing [25]. Moreover, common regents used are derived from fungus [26]. The presence of fungal DNA in the enzyme Zymolyase and 10 X PCR buffer has been reported [26]. Thus, reagents should be carefully selected and any new lots were checked before use to avoid contamination. In this study the use of lyticase (Sigma), a synthetic equivalent of β-1,3-glucanase, avoids the impurities found in zymolyase [26, 27]. With the development of new procedures with improved sensitivities, the problem may become more apparent. It is therefore essential that the specificities of assays be vigorously confirmed. The actual source of fungal nucleotides in the blood is still unknown. Whether they are extracted from intact cells (including nonviable/non-culturable cells) circulating in the blood stream or from fungal cells that have been engulfed by white blood cells, or from free mucleic acid, remains unclear [28]. Thus, we extract fungal DNA from buffy-coat layer of whole blood containing intact fungal cells in stead of free mucleic acid in the serum.

4. Pan-fungus vs. species-specific detection. Although *Candida* and *Aspergillus* were most important human pathogens, other fungi emerge in

recent decade. As *Aspergillus, Zygomycetes* and several fungi were rarely, if any, isolated in current blood culture systems, pan-fungus amplification was warranted. However, during previous study we found that current amplification techniques based on the use of pan-fungal primers have limitations: contamination, and similarity between the sequence of the primers or probes and human DNA (data not shown).

5. Sensitivity vs. specificity. Oligonucleotide primers were carefully selected to amplify fungal DNA of interest and avoid nonspecifically amplification of human genomic DNA. However, in order to increase sensitivity, the amount primers added in much higher than those for PCR assays for bacteria and virus. Non-specific bands occurred which hampers the clinical application of PCR assays in routine use as both human and fungi are eukaryotes.

Recently, qualitative real-time PCR systems have been developed for the detection of fungal pathogens, such as *Candida* sp., *C. neoformans*, and *Aspergillus* sp. All assays demonstrate sensitivities better or comparable to previously described PCR methods [29]. Both amplification and detection are carried out in one tube; hence the need for subsequent gel electrophoresis or hybridization is obviated. Real-time PCR assays dramatically decrease the risk of false-positive results, because both the PCR reaction and detection system are coupled and conducted in closed glass capillaries, which can avoid the possibility of contaminating amplicons from the environment.

The ITS regions are located between the 18S and 28S rDNA genes and offer distinct advantages over other molecular targets including increased

sensitivity due to the existence of approximately 100 copies per genome. By analysis of ITS1 [30-32] or ITS2 [33, 34] fragment lengths variation, many *Candida* species can be differentiated. The Light Cycler system offers another advantage of analysis of melting temperature of amplicons. The melting temperature of the amplicon is dependent on the G+C content, sequence length and base composition. Our previous data showed that the Light Cycler technique is capable of rapidly identifying fungal species with high sensitivity and offers potential for quantitative results [23]. Each of the specific primers selectively and exclusively amplified its own DNA fragment from the corresponding genomic DNA of the fungal species. Furthermore, in our study, each fungal species has a characteristic Tm, which helps to further confirm the identity of fungal species.

(5)結論與建議

As such a very small amount of fungal DNA would be present in the blood samples obtained from patients with invasive fungal infections [10], the sensitivity of the assay is critical. In this study we further confirmed that the Light-Cycler system was able to detect as little as 1 cfu/mL of blood without nested PCR or probe detection. To apply this PCR assay in the clinical practice, several issues were important and should be solved. In conclusion, the real-time Light-Cycler PCR assay combines rapid *in vitro* amplification of DNA with real-time species determination and quantification. This method is simple, rapid, sensitive, and cost-effective and seems to be very promising.

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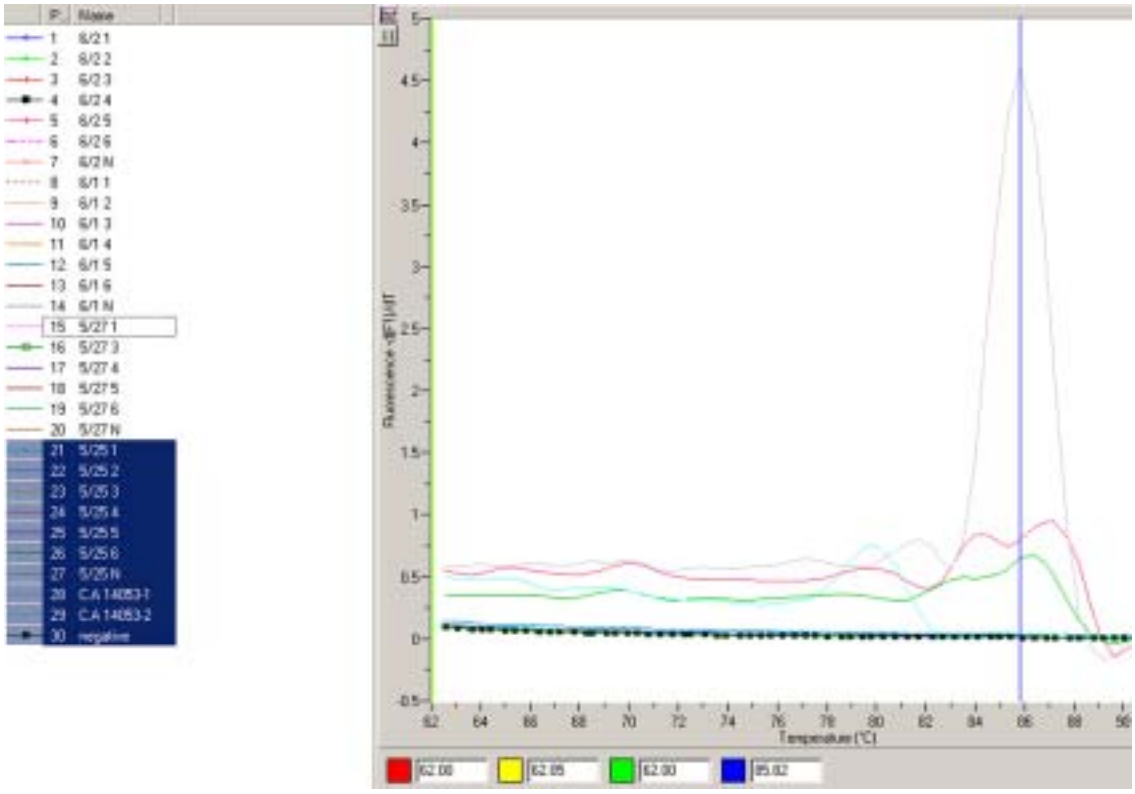
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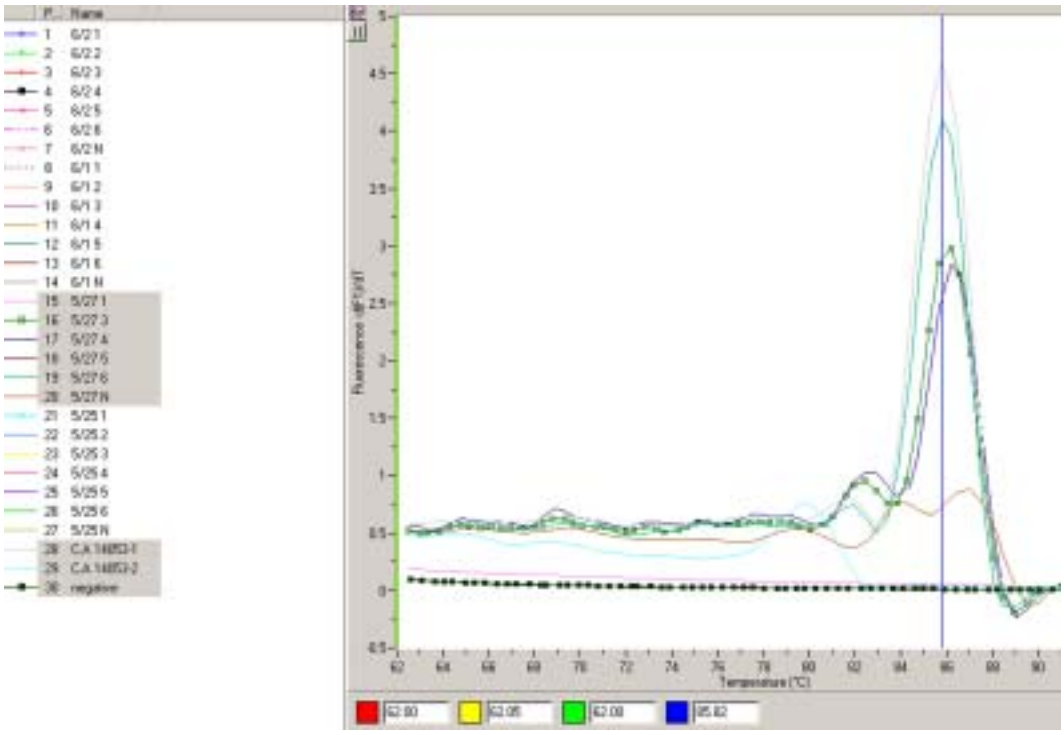

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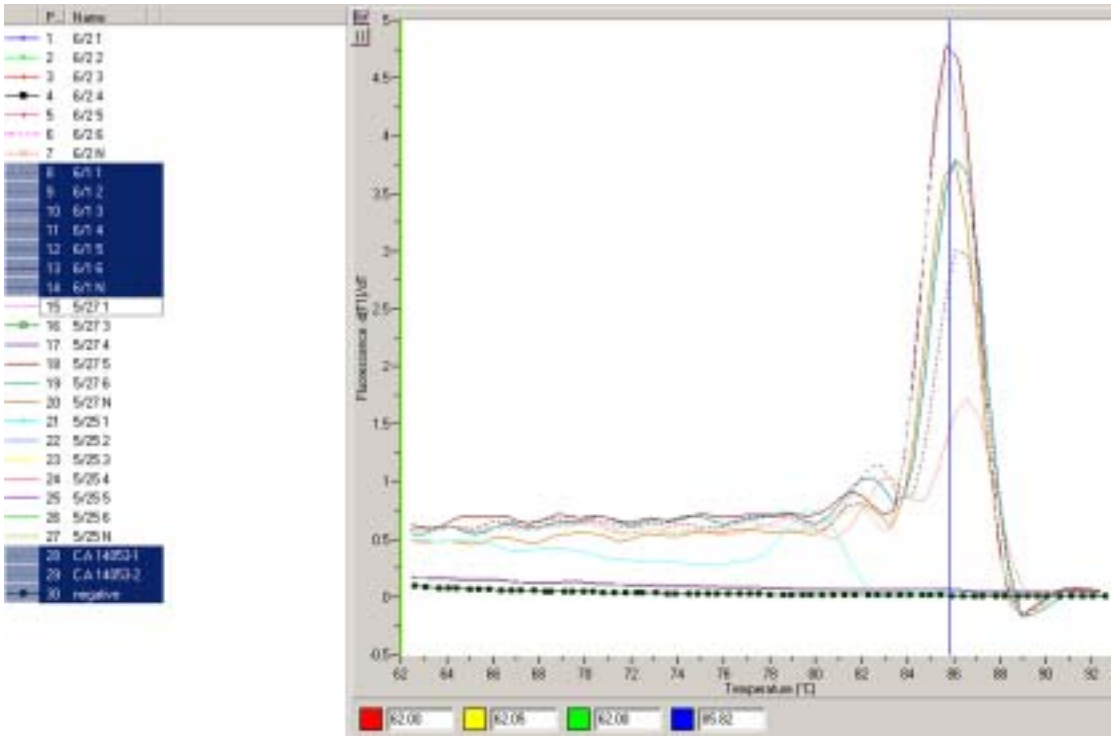

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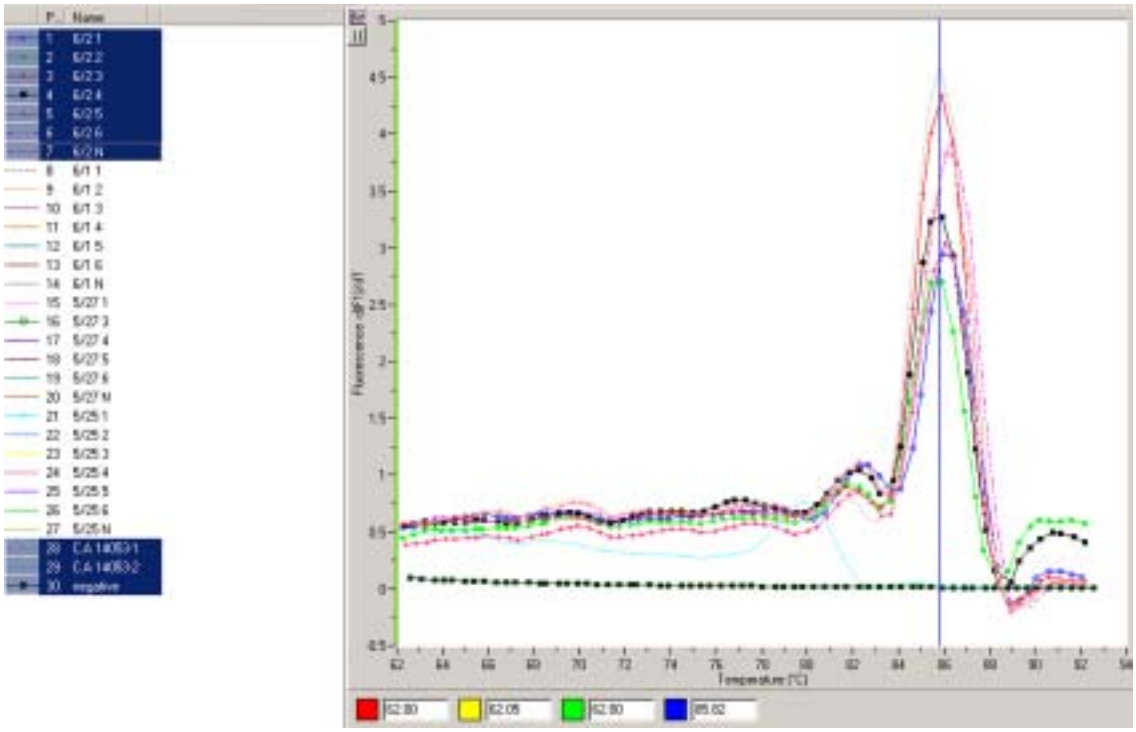

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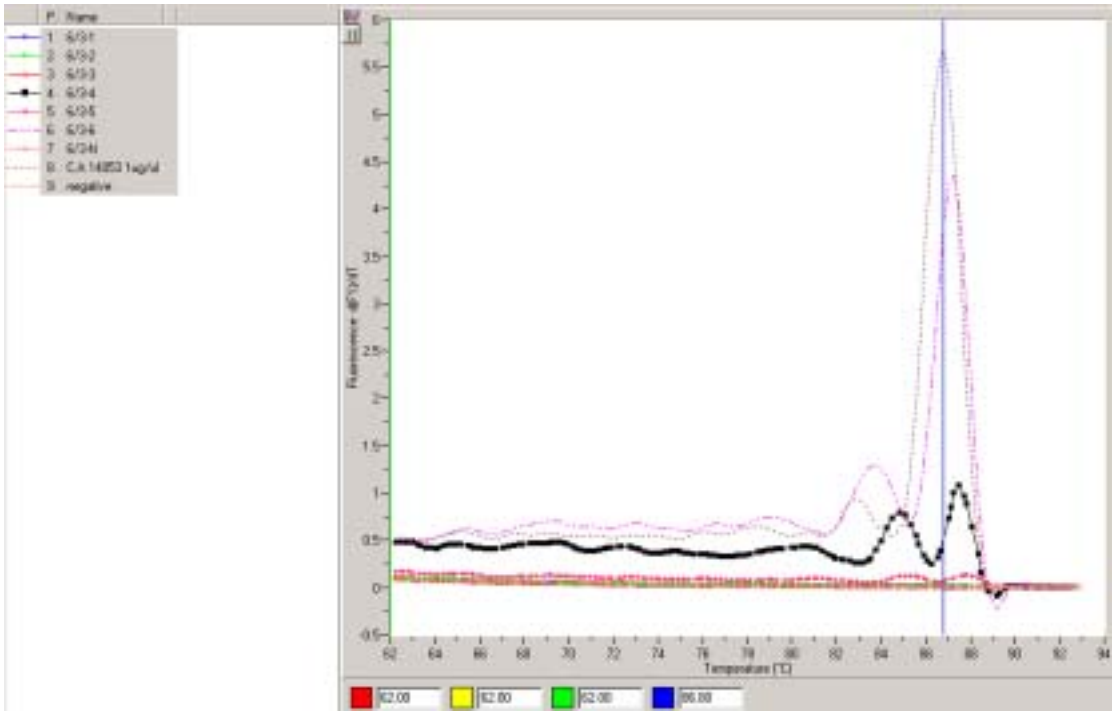

(E)

Fig. 1(A)~(E). The sensitivity of the Light-Cycler system to detect *Candida albicans* in blood. Peripheral blood samples from healthy donors were spiked with defined numbers of *C. albicans*. Characteristic dose-dependent melting peaks were observed in the presence of *C. albicans* DNA derived from 10^1 to 10^6 colony-forming units per mL of blood.

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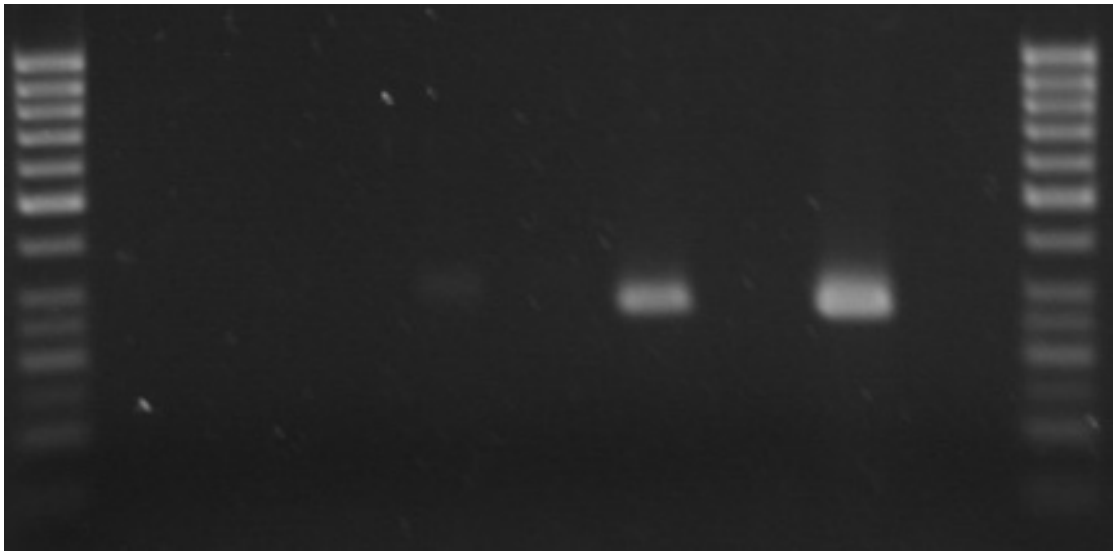

M 1 2 3 4 5 6 N P N

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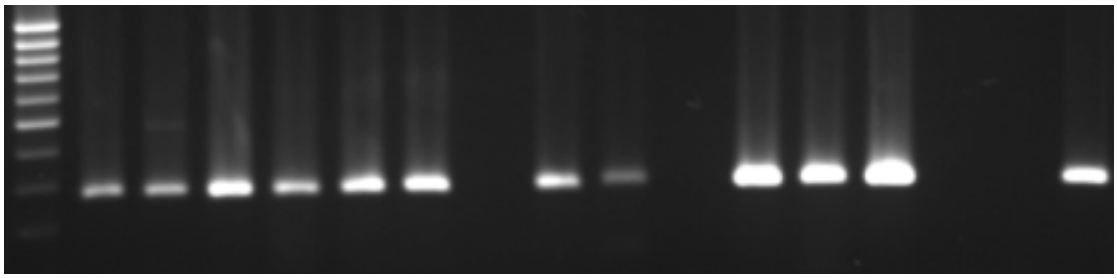

M 1 2 3 4 5 6 N 1 2 3 4 5 6 N 1 3

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Fig. 2 The sensitivity of the Light-Cycler system to detect *Candida albicans* in blood in agar gel electrophoresis.

九十三年度計畫重要研究成果

計畫名稱：利用 Real-Time Light-Cycler PCR 技術快速鑑定高危險群患者血液中的病原真菌-III

主 持 人：陳宜君　　計畫編號：DOH93-DC-1102

1.計畫之新發現或新發明

建立快速、敏感之檢驗方法，以協助判定侵襲性黴菌感染，希望能改善病人的預後並避免昂貴的抗黴菌藥物濫用。

2.計畫對民眾具教育宣導之成果

無

3.計畫對醫藥衛生政策之具體建議

無