行政院國家科學委員會補助專題研究計畫成果報告

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※　以 nested PCR 偵測血液中麴菌 DNA 之存在　※

※　Detection of *Aspergillus* spp. in blood by　※

※　nested PCR　※

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□國際合作研究計畫國外研究報告書一份

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Abstract.

The increasing incidence of aspergillosis and other invasive fungal infection, a life-threatening infection in immunocompromised patients, emphasized the need to improve the currently limited diagnostic tools. We have established a nested PCR assay that specifically amplified an inter-spacer (ITS2) region between the multicopy 18S rRNA gene that is highly conserved in fungus species. Methods for extraction of DNA of yeast or mold from human blood was determined which was designed to isolate DNA from intact fungi (rather than free DNA) and to avoid contamination from common reagents. To apply this PCR assay in the clinical practice, several issues were important and discussed. In the following year, a prospective cohort study will be conducted in hemato-oncology wards to evaluate clinical value of PCR-based diagnostic method for early diagnosis of invasive fungal infection in high-risk patients.

Introduction

Invasive pulmonary aspergillosis is a life-threatening complication of intensive chemotherapy for malignancies, chronic treatment with high dose corticosteroids, transplantation, and AIDS. 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 aspergillosis [2]. Therefore, alternative strategies are needed for the early recognition of invasive aspergillosis to allow better selection of patients who need treatment, while reducing the number of patients who are exposed unnecessarily to these drugs and their side effects. In addition, better selection of patients who require treatment for invasive aspergillosis will also help to contain the cost of treatment when novel and expensive drugs are used [6]. Furthermore, increasing use of amphotericin B raise the concern of emergence of resistance and alternative antifungal agent is limited, if any, at present time.

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 aspergillosis has faced several aspects of difficulties. Fist, 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 [9,10]. 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 [10]. 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 [11]. 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 [12].

Materials and Methods

Stains tested for species specificity of PCR assay. The following fungal standard strains will be used in the study, *A. fumigatus, A. flavus, A. terreus, A. niger, Candida albicans, C. tropicalis, C. glabrata, C. parapsilosis,* and *C. krusei.*

Collection of clinical specimens. Blood samples will be collected from the high-risk adult patients which include acute leukemia, chronic myelogenous leukemia with blast crisis, advanced myelodysplastic

syndrome (refractory anemia with excess of blasts, or blast transformation) while under antileukemic therapy, advanced or relapsed high- or intermediate-grade malignant lymphoma, advanced or relapsed Hodgkin's disease, or advanced chronic lymphocytic leukemia. Human immunodeficiency virus-positive patients were not included. Criteria for inclusions of high-risk patients will be neutropenia (granulocyte count < $1.0x10^9$/liter), fever (body temperature >38.3°C), unresponsiveness to the first-line antibacterial treatment, and/or newly arisen nonspecific pulmonary infiltrates or pulmonary nodules proven by conventional chest radiography. High-resolution computed tomography (HR-CT) scans of the lung will be performed by standardized techniques by the Department of Radiology, NTUH. Small angiotropic round infiltrates, halo signs around infiltrates, or wedge-shaped small peripheral lung infarctions will be judged as typical early pulmonary aspergillosis findings, the "air crescent sign" will be considered a sign of a later disease stage. The HRCT scans were performed, and the findings will be analyzed by a radiologist.

Blood samples were obtained under sterile conditions by venipuncture, usually simultaneously with blood samples for culture for microbiological examination. The sample volume will be 5 ml.

DNA extraction from blood samples. 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. Blood samples were processed within 2 hr of venipuncture. leukocytes and fungal cells floating in the blood or found within human leukocytes were separated from erythrocytes by Ficoll-Hypaque gradient method and were pelleted by centrifugation at 1,500 x *g*. Incubation 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, the samples were pelleted and incubated with 50 mM NaOH at 95°C for 10 min. Neutralization with 1 M Tris (pH 7.0) was followed by treatment with 300 μg of Zymolyase (ICN, Costa Mesa, Calif.) per mL in 50 mM Tris (pH 7.5), 10 mM EDTA, and 28 mM β–mercaptoethanol at 37°C for 45 min to give plasts. After centrifugation at 5,000 x *g* the supernatant containing human DNA and proteins was decanted and the pellets were treated with 1 M Tris-EDTA and 10% SDS at 65°C for 30 min for plast lysis. Then, 5M potassium acetate was added and the samples were incubated at –20°C for 30 min for protein precipitation. After an additional centrifugation step at 1,000 x *g* for 20 min, DNA precipitation from the supernatant was carried out with cold isopropanol. DNA was purified with 70% ethanol, air dried, and resuspended in 40 ul of H_2O.

DNA extractrion from fungal suspension. Fungal isolates were cultures on Sabouraud medium at 30C; *Candida* isolates were cultured for 48 h, and *Aspergillus* isolates were cultured for 72 h. Thereafter, fungal saline suspensions were adjusted photometrically (A_{530}; McFarland no. 0.5 standard) to a concentration of $1x10^6$ to $5x10^6$ cells/mL. Tenfold serial dilutions (10^6 to 10^0 cells) were prepared to test the sensitivity and specificity of the assays. The fungal suspension with predetermined numbers of CFU per ml were centrifuged at 5,000 x *g*, and then the pellet was incubated with 5 ml of leukocyte lysis buffer. The following procedures were as mentioned above.

Oligonucleotide primers and PCR conditions. The oligonucleotide primers used in this study were based on the comparison of the sequences of 18S rDNA genes of *Aspergillus* species and other fungi deposited in the GenBank database (Fig. 1). A nested, two-step PCR will be performed with two sets of primers [14,15]. By using a nested, two-step PCR technique, different primers was tested. As an internal control, a 138-bp PCR fragment encoded by the human glycose-6-phosphate dehydrogenase gene (GeneBank accession no. X55448) was amplified

with primers G6PD1S and 1AS in each clinical sample. Control samples included all the constituents in the reaction mixture except genomic DNA. As negative and positive PCR controls, DNA from the human blood or dilute samples of *A. fumigatus*, respectively, was used as templates. The PCR products were separated by 2.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized with UV light.

Comtamination precautions. Precautions were taken to avoid possible contamination of PCR samples by following the guidelines of Kwok and Higuchi [17] 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.

Determination of sensitivity of nested PCR for blood samples. The sensitivity of the PCR assay with the optimal two pairs of nested oligonucleotides were determined by spiking blood samples from healthy volunteers with serial dilutions of fungal conidia and performing similar nested PCR amplification assays.

Results

Sensitivity of two-step PCR. The sensitivity of the two-step PCR assay with purified *Candida albicans* DNA was shown in Fig. 2. The signal derived from 100 pg of *C. albicans* template DNA was detectable by ethidium bromide staining of an agarose gel. To determine the sensitivity of this PCR assay in blood samples, peripheral blood samples from healthy donors were spiked with defined numbers of *C. albicans*. The signal derived from 10^3 CFU per ml of blood was detectable by ethidium bromide staining of an agarose gel (Fig. 3).

Genus and species specificity of PCR assays. Using universal fungal primers ITS3-ITS4 and ITS86-ITS4 in the two-step PCR, the second step (with ITS86-ITS4) amplifies an internal fragment of ITS2 of 279-bp from *C. albicans*, 251-bp from *C. parapsilosis*, 360-bp from *C. glabrata*, 269-bp from *C. tropicalis*, 282-bp from *C. krusei*, 284-bp from *A. fumigatus*, 306-bp from *Cryptococcus laurentii*, and 350-bp from *Cryptococcus albidus* (see Fig. 4).

Detection of fungal DNA in blood samples. Our preliminary data showed fungus DNA was detected in blood samples collected from a leukemic patients with persistent fungemia due to C. parapsilosis (sample no. 7), and 3 patients with persistent febrile neutropenia despite appropriate antibacterial therapy for 5 days (Fig. 5).

Discussion

Our preliminary study demonstrated that before application of fungal PCR assays into the "real world", several issues should be considered.

1. **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 [9,11], and the difficulty in releasing fungal DNA in the preparation of clinical specimen due to the cell wall structure of the fungi.
2. **Contaminations occurring in fungal PCR assays.** Contaminations occurred during blood sampling as well as processing. Moreover, common regents used are derived from fungus. Thus, reagents were carefully selected and any new lots were checked before use to avoid contamination.
3. **Pan-fungus vs. species-specific detection.** Although *Candida* and *Aspergillus* were most important human pathogens, other fungi emerge in recent decade. As *Aspergillus* and several fungi were rarely, if any, isolated in current blood culture systems, non-culture system for panfungus was warranted. However, Aspergillus and other mold are common in our environments. Current PCR assays for either panfungus or specific species are not ideal due

several reasons.

4. **Sensitivity vs. specificity.** Oligonucleotide primers were carefully selected to amplify fungal DNA of interest and avoid nonspecifically amplify 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.

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Table 1. Primer sequences and location.

Primer	Nucleotide sequence (5' to 3')	Location	Reference
Fungus 5.8S rDNA			
ITS3	GCA TCG ATG AAG AAC GCA GC		16
ITS86	GTG AAT CAT CGA ATC TTT GAA C		23
Fungus 28S rDNA			
ITS4	TCC TCC GCT TAT TGA TAT GC		16
***Aspergillus fumigatus* 18S rDNA (GenBank accession no. AB008401)**			
AFU5S	AGG GCC ATC GAG TAC ATC ACC TTG	1436-1459	15
AFU5AS	GG G (AG)G CGT TGC CAA C(CT)C (CT)CC TGA	1648-1771	15
AFU7S	CGG CCC TTA AAT AGC CCG	1296-1313	15
AFU7AS	GA CCG GGT TTG ACC AAC TTT	1681-1700	15

* Nucleotides in parentheses are degenerate.