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Original Article

Rapid identification of invasive fungal species using sensitive universal primers-based PCR and restriction endonuclease digestions coupled with high-resolution melting analysis

Ming-Horng Tsai [a,d,f], Lee-Chung Lin [c], Jen-Fu Hsu [b,d], Mei-Yin Lai [b,d], Hsuan-Rong Huang [b,d], Ming-Chou Chiang [b,d], Jang-Jih Lu [c,e,*]

[a] *Division of Neonatology and Pediatric Hematology/Oncology, Department of Pediatrics, Chang Gung Memorial Hospital, Yunlin, Taiwan*
[b] *Division of Pediatric Neonatology, Department of Pediatrics, Chang Gung Memorial Hospital, Taoyuan, Taiwan*
[c] *Department of Laboratory Medicine, Chang Gung Memorial Hospital at Linkou, Taoyuan, Taiwan*
[d] *College of Medicine, Chang Gung University, Taiwan*
[e] *Department of Medical Biotechnology and Laboratory Science, Chang Gung University, Taoyuan, Taiwan*
[f] *Graduate Institute of Clinical Medical Science, College of Medicine, Chang Gung University, Taiwan*

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Abstract *Backgound:* Conventional diagnosis of invasive fungal disease from blood cultures is often notoriously delayed and inadequately sensitive. We aimed to develop a universal primers-based polymerase chain reaction (PCR) assay and restriction fragment length polymorphisms (RFLP) for rapid identification of invasive fungal disease (IFD).
Methods: We evaluated 16 clinical fungal species using a combination of PCR assays with 3 different restriction endonucleases targeting various internal transcribed spacer (ITS) regions and high resolution melting analysis (HRMA). Serial samples from 75 patients suspected to have IFD were analyzed for clinical verification.

List of abbreviation: CGMH, Chang Gung Memorial Hospital; HRMA, high resolution melting analysis; ITS, internally transcribed spacer; SD, standard deviation; IFD, invasive fungal disease; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

* Corresponding author. Department of Laboratory Medicine, Chang Gung Memorial Hospital, 5, Fu-Shing St., Kwei-Shan, Taoyuan, 333, Taiwan. Fax: +886 3 3971827.
E-mail address: janglu45@gmail.com (J.-J. Lu).

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Results: We have designed a universal PCR capable of amplifying a portion of the 18S rRNA gene of 16 clinically important fungal species. The restriction patterns of most PCR products generated by *Eco*RI or double digested by *Cla*I and *Ava*I were different, except *Aspergillus niger* and *Aspergillus flavus* had a similar pattern, and *Aspergillus fumigatus* and *Aspergillus terreus* had a similar pattern. All these species had a unique melting curve shape using the HRMA. Both HRMA and universal PCR had adequate sensitivity, and all sixteen reference fungal species can be clearly distinguished by the universal PCR-RFLP-HRMA assay. With a reference library of 176 clinically relevant fungal strains, and 75 clinical samples from patients with suspicious IFD were tested, our assay identified 100% and 61.1% of isolates from the reference library and clinical samples, respectively.
Conclusions: Universal PCR and RFLP coupled with HRMA could be a highly discriminative and useful molecular diagnostic that could enhance the current diagnostic, treatment, and surveillance methods of invasive fungal disease.

Introduction

Blood and/or sterile-site culture is the existing standard for the diagnosis of invasive fungal infection, despite the identification of fungal species taking more than 48—96 h and the sensitivity being notoriously inadequate.[1,2] It has been reported that blood cultures are negative for *Candida* species in approximately 30—50% of autopsy-proven cases with disseminated candidiasis.[3,4] Indeed, poor outcomes of invasive candidiasis result from delayed or missed diagnosis, especially in immunocompromised patients or in the intensive care unit.[5—7] Furthermore, the initial signs and symptoms of invasive fungal disease are non-specific and indistinguishable from bacterial infection in the first 48—72 h, when ineffective or unnecessary broad-spectrum antibiotics are often administrated.[8—10] Therefore, various techniques have been proposed as an alternative to microbiological cultures in recent years.[1,11—16]

The β-D-glucan (BDG) Fungitell Assay and the detection of *Candida albicans* germ tube antibody (CAGTA; *C. albicans* IFA IgG, Vircell, Granada, Spain) are both widely used as an adjunct in the diagnosis of invasive candidiasis, but these tests come after a presumptive diagnosis or when the clinical suspicion is already present.[13,14,16] Developing DNA or antigen-based detection for early fungal species identification is now the highlight of the mold research.[16] Polymerase chain reaction (PCR) assays specifically targeting regions of the 5.8S, 18S and 28S rRNA genes, as well as internally transcribed spacer (ITS) regions have been developed for the detection of *Candida* spp. in various populations.[15,17—19] They reported a pooled sensitivity and specificity of >85% in a meta-analysis.[19] Another strategy is based on high resolution melting analysis (HRMA), which has already been applied in bacteriology,[20,21] virology[22,23] and mycology.[24,25] However, these approaches rarely can differentiate multiple fungal species simultaneously and lack verified comprehensive databases for the uncommon species. The objectives of this study were to develop, evaluate and validate a new combined HRMA and PCR assay with universal primers and restriction endonuclease digestions for early diagnosis of invasive fungal disease.

Materials and methods

Strains

Sixteen strains of important fungal species that are most frequently isolated in human infections, either from an international collection (Centraal bureau voor Schimmelcultures [CBS], Utrecht, Netherlands; American Type Culture Collection [ATCC], Manassas, Virginia, USA), or from the laboratory collection in the National Cheng Kung University affiliation Hospital (Mycology laboratory) [BCRC], were used in this study, as follows: *C. albicans* CBS 562, *Candida krusei* BCRC 20514^T, *Candida tropicalis* BCRC 20520^T, *Candida parapsilosis* BCRC 20515^T, *Candida guilliermondii* ATCC 20403, *Candida lusitaniae* CBS 6936, *Candida glabrata* BCRC 20586^T, *Cryptococcus neoformans* CBS 132, *Trichosporon asahii* CBS 2479, *Aspergillus niger* ATCC 16888, *Aspergillus flavus* BCRC 30007, *Aspergillus fumigatus*, BCRC 30502^T, *Aspergillus terreus* BCRC 31128, *Fusarium solani* CBS 109028, *Scedosporium apiospermum* ATCC 44329, and *Scedosporim prolificans* CBS 494.92. All of above reference strains were also collected for use in our HRM analysis.

DNA extraction and nucleotide sequence determination

Yeasts were recovered from Sabouraud dextrose agar and/or Brain Heart Infusion agar. The fungal DNA was extracted using QIAamp DNA Mini Kit (Qiagen®, Germany) according to the manufacturer's recommendations. Clinical isolates sequencing was only carried out in cases when the HRMA pattern did not correspond with any reference strain, some inconsistencies in HRMA pattern were noted, or the phenotype was not determined unambiguously. Amplicons of PCR (see below) were purified and sequences from both DNA strands were generated and edited with Sequencher™ version 4.9 (Gene Codes Corporation), followed by alignment using Mega version 4.0.2 software.

Clinical specimens

From April 2003 to December 2014, 175 clinical isolates obtained from different hospitalized patient who had documented invasive fungal infection in the CGMH, confirmed by the API 32C AUX yeast identification kit (bioMérieux SA, Marcy l'Étoile, France) and chromogenic culture media (CHROMagar, Becton Dickinson and Company, USA) were examined for the verification of the universal PCR coupled with HRMA developed in this study. These clinical isolates were previously stored at −20 °C in the central laboratory of CGMH. Furthermore, we tested the clinical specimens prospectively collected from patients with suspicious invasive fungal disease in CGMH between March 2016 and August 2016. These clinical samples were examined for the presence of fungi by both culture and the universal PCR-HRMA assay developed in this study. The study was approved by the Institutional Review Board and Human Research Ethics Committee of CGMH with informed consent obtained from all patients.

PCR-restriction fragment length polymorphism (RFLP)

After extraction of the fungal DNA, PCR amplification targeting the *ITS1* and *ITS4* regions were performed using 1 μl template DNA, 10 μl Phusion Flash PCR Master Mix (Thermo®#F548), 2 μl primer mix (4um for each primer: U1: 5′-TCCGTAGGTGAACCTTGCGG-3′ and U2: 5′-TCCTCCGCTTATTGATATGC -3′), and 7 μl sterile water up to final volume of 20 μl. The PCR program consisted of an initial denaturation of 1 min at 98 °C, followed by 29 cycles of 1 s at 98 °C, 5 s at 52 °C and 15 s at 72 °C, and ended with 1 min at 72 °C. Five microliters of each PCR product was digested with different restriction enzymes in appropriate restriction enzyme buffer in a total volume of 20 μl. After incubation with *Eco*RI (NEB#R0101), *Ava*I (NEB #R0152) and *Cla*I (NEB # NEB #R0197) at 37 °C for one hour, the digested DNA was electrophoresed on a 6% polyacrylamide gel.

Polymerase chain reaction (PCR) and high resolution melting analysis (HRMA)

PCR amplification and HRMA of the ITS2 region were performed on the LightCycler® 480 (Roche) with primers (8a: 5′-CATGCCTGTTTGAGCGTC-3′, and 2b: 5′CTACCCGCTGAACTTAAGCAT-3′). PCR was performed with LightCycler® 480 High Resolution Melting Master (Roche Diagnostics, Meylan, France), 20 ng of fungal DNA, 3.0 mmol/l $MgCl_2$ (Roche), 1.5 × saturating dye EvaGreen (Biotium, USA), 0.35 μmol/l of each primer (final concentration) and sterile water up to 20 μl.

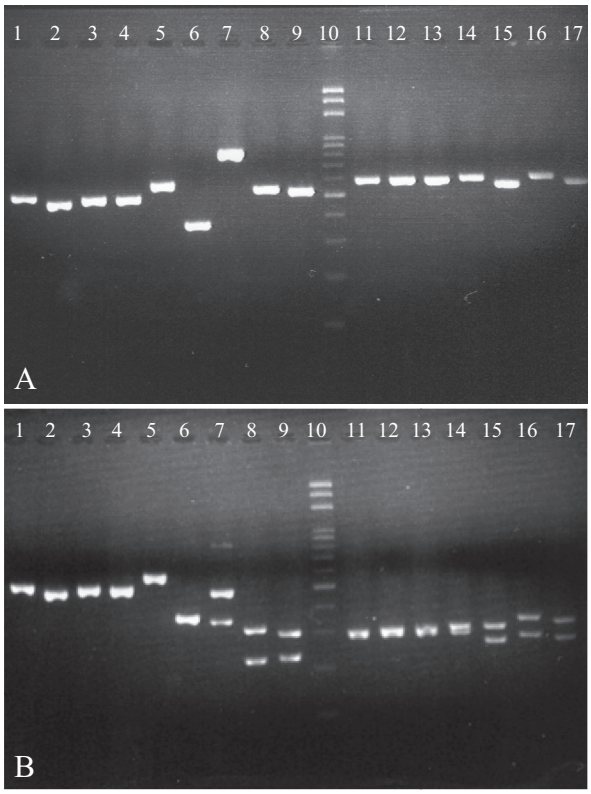
The PCR program consisted of an initial denaturation of 10 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, 10 s at 58 °C and 10 s at 72 °C, and ended with 4 min at 72 °C. The amplification products for HRMA (137—299 bp long with GC content 39—58%) were cooled 1 min at 40 °C and then heated from 65 °C to 95 °C monitoring fluorescence at the rate of 0.02 °C/s. Melting data were normalized and the temperature shifted using LightCycler® 480 software version 1.5 (Roche Diagnostics, Meylan, France). Normalization regions for HRMA were at 79—81 °C and 87—89 °C. All reference strains were analyzed in duplicates in 3 independent runs and the mean Tm and SD values were calculated for each melting domain. Different Tm value ranges were tested and finally the mean Tm ± 3SD range was selected to overcome the interassay variability and reach maximum sensitivity and reproducibility.

Results

DNAs from the sixteen fungal reference strains were examined by the universal PCR. All of these DNA samples generated a PCR product of the expected and similar sizes (510—613 bp) except a significant large size (885 bp) and a small size (386 bp) for *C. glabrata* and *C. lusitaniae*, respectively (Fig. 1A). The PCR products were continuously digested with either *Eco*RI (Fig. 1B) or *Ava*I and *Cla*I (Fig. 1C) to determine possible restriction fragment length polymorphism to distinguish certain fungal species. Our data showed only C. glabrata has been digested by *Eco*RI (Fig. 1B), and all non-*Candida* species had two significantly smaller fragments (between 265 bp and 337 bp) after *Eco*RI digestion which were suitable for distinguish *Aspergillus* species. All *Candida* species and non-*Aspergillus* species had different pattern under *Ava*I plus *Cla*I digestion. For *Aspergillus* species, *A. niger* and *A. flavus* had a very similar pattern, and *A. fumigatus* and *A. terreus* had a very similar pattern. All restriction fragment length polymorphism pattern was listed in Table 1, and 175 clinical isolates were examined, including 22 isolates of *C. albicans*, 21 isolates of *C. krusei*, 21 isolates of *C. guilliermondii*, 21 isolates of *C. glabrata*, 22 isolates of *C. tropicalis*, 9 isolates of *C. parapsilosis*, 7 isolates each of *C. metapsilosis* and *C. orthopsilosis*, 12 isolates of *C. lusitaniae*, 18 isolates of *Cryptococcus neoformans*, 2 isolates each of *T. asahii*, *A. niger*, *A. flavus*, *A. fumigatus*, *A. terreus*, *F. solani*, *S. apiospermum*, and *S. prolificans*. Our data showed that all of the clinical isolates have the same digestion patterns as their reference strains.

To determine the sensitivity of this universal PCR, *C. albicans* CBS 562 and *C. parapsilosis* BCRC 20515^T cultures were serially diluted in a serum sample that was confirmed to be PCR negative. An aliquot of each dilution was subjected to DNA isolation, and the purified DNA was used as the template for the PCR. The end point of the universal PCR for *C. albicans* DNA was found to be approximately 21 organisms. The sensitivity for detection of *C. parapsilosis* was determined to be approximately 250 organisms. These experiments were repeated three times, and the results from all three runs were the same.

In order to distinguish species with similar RFLP pattern, HRMA were performed with 16 fungal reference strains and showed reproducible melting peaks for most species (Fig. 2). Based on the relative signal shifting mode, *Candida* species can be divided into two different groups. C. albican, C. parapsilosis, C. tropicalis, and C. lusitaniae exist quickly drop signal at specific temperature range, and then smoothly decreasing after that specific temperature range (Fig. 2a). The specific temperature for *C. albicans* was 84.6 °C — 85.8 °C, *C. parapsilosis* was 81.4 °C — 83.6 °C, *C. tropicalis*



1. *Candida albicans*
2. *Candida krusei*
3. *Candida tropicalis*
4. *Candida parapsilosis*
5. *Candida guilliermondii*
6. *Candida lusitaniae*
7. *Candida glabrata*
8. *Cryptococcus neoformans*
9. *Trichosporon asahii*
10. Marker
11. *Aspergillus niger*
12. *Aserpgillus flavus*
13. *Aspergillus fumigates*
14. *Aspergillus terreus*
15. *Fusarium solani*
16. *Scedosporium apiospermum*
17. *Scedosporium prolificans*

Figure 1. U1—U2 PCR products of all 16 clinically important fungal species (A) and the *EcoR*I digestion patterns (B). The size of the molecular size standards are marked on the middle of the gel.

was 80.2 °C — 82.4 °C, and *C. lusitaniae* was 85.0 °C — 87.0 °C. *C. glabrata*, *C. guilliermondii*, and *C. krusei* exist twice quickly drop signal process; *C. glabrata* was 79 °C — 83 °C and 84.4 °C — 85.8 °C; *C. guilliermondii* was 82.8 °C — 84 °C and 85 °C — 86.4 °C; and *C. krusei* was 77.0 °C — 83.5 °C and 89.2 °C — 90.8 °C, respectively. The HARM analysis of four Aspergilus species varied, only A. fumigatus exist distinct twice quickly drop signal process on 76.0 °C — 81.5 °C and 85.0 °C — 86.4 °C (Fig. 2b). The signal of *A. terreus* was decreased to nearly 40% after temperature was higher than 81.0 °C — 82.0 °C, which can be distinguished from *A. fumigatus*, *A. niger* and *A. flavus*. The pattern of dMelt curve between *A. niger* and *A. flavus* were too diverse to differentiate each other. Therefore, *Cla*I plus *Ava*I digested pattern was needed to discriminate these two species.

To evaluate the universal PCR-RFLP process, 75 clinical samples prospectively collected from patients with suspicious invasive fungal disease was examined. These 75 clinical samples included 45 from bronchoalveolar lavage, 17 from pleural fluid, 12 from cerebrospinal fluid, and 1 from synovial fluid. Among them, 18 of these specimens had positive cultures among these 16 fungal species (12 from bronchoalveolar lavage, 3 from pleural fluid, and 3 from cerebrospinal fluid), and 11 (61.1%) of them were concordance to the universal PCR-RFLP assay (Table 2). All of these 11 clinical specimens generated positive PCR results, and then were digested with *EcoR*I. The results were identical to those of the reference strains. None of the remaining 57 clinical specimens produced positive PCR result, except 9 had positive cultures for other uncommon fungal species (Table 2). Specimens from all of the nine clinical isolates generated unique dMelt curve, and identification of species level can be achieved after universal PCR with RFLP pattern.

Discussion

In this study, we designed one set of PCR primers based on the conserved sequence of the ITS regions or the 18S rDNA genes of various fungus. The universal PCR products from different fungal species were found to have different restriction patterns after digestion with either *EcoR*I or *Ava*I plus *Cla*I. The poorly differentiated RFLP patterns between four *Candida* species can be solved by the distinguishable HRMA patterns. Furthermore, the PCR products from different isolates of the same fungal species had the same restriction pattern, which concluded this PCR assay discriminative. The differentiation capacity of the 18S PCR coupled with HRMA for 16 fungal species, making up the majority of clinical important mold isolates, was analyzed in this study. The assay time of this diagnostic strategy takes an average of 6 h and can provide at least a 26-h time-saving than the traditional identification methods require. Most importantly, more than 90% of these PCR-RFLP identifications were concordant with the culture results at the species level.

In contrast to previous rapid PCR-based platforms designed for detection of a limited number of fungal species,[12,18] our PCR-RFLP allows the simultaneous detection and identification of much more various *Candida* and *Aspergillus* species. A similar strategy of universal PCR and

Table 1 Universal PCR and *EcoRI* digested RFLP pattern of 16 reference fungal species.

Fungal Species	ITS1-ITS4 PCR product size (bp)	*EcoRI* digested PCR fragment size (bp)
Candida albicans CBS 562	536	536
Candida krusei BCRC 20514T	510	510
Candida tropicalis BCRC 20520^T	524	524
Candida parapsilosis BCRC 20515^T	520	520
Candida guilliermondii ATCC 20403	607	607
Candida lusitaniae CBS 6936	386	386
Candida glabrata BCRC 20586^T	885	515, 370
Cryptococcus neoformans CBS 132	555	325, 230
Trichosporon asahii CBS 2479	541	309, 232
Aspergillus niger ATCC 16888	599	305, 294
Aserpgillus flavus BCRC 30007	595	305, 290
Aspergillus fumigates BCRC 30502^T	596	304, 292
Aspergillus terreus BCRC 31128	608	314, 294
Fusarium solani CBS 109028	566	308, 258
Scedosporium apiospermum ATCC 44329	613	337, 276
Scedosporium prolificans CBS 494.92	585	320, 265

restriction endonuclease digestions has been developed for identifying common bacterial pathogens in the cerebrospinal fluid.[26] Additional advantages of this universal primer PCR-RFLP are the lower cost, simple conduction, and easy to interpret. After the practical utility has been evaluated by a clinical test trial, it is possible to shorten the delay of appropriate antifungal treatment, which is usually started after 2—3 days of unresponsiveness to antibiotics in high-risk patients.[10,19]

Because the efficiency of PCR-based amplification of fungal DNA is dependent on the specificity of the primers, we make use of the characteristics of ribosomal RNA (rRNA) gene cluster, which contains the 18S, ITS1, 5.8S, ITS2 and 28S regions and the sensitivity can be significantly increased due to its multiple copies existing in fungal cells.[27] The primers that can bind to the conserved areas and hypervariable areas of the fungal DNA sequences make the assay applicable to identify $>$ 1 fungal genera and detect specific fungal species, respectively.[28] Therefore, our primers, based on the above principle, were designed to target the conserved regions of 5.8S and ITS1 sequences of 16 potential fungal pathogens.

A recent meta-analysis of the diagnostic accuracy of PCR directed in blood samples showed that the sensitivity and specificity values were 93% and 95% for the diagnosis of invasive candidiasis.[29] Among these various PCR-based methods for diagnosis of invasive candidiasis, there were marked differences in the nature of the samples (whole blood containing intact blood cells versus serum or plasma, fresh versus frozen, stored blood samples), PCR techniques (standard, nested, or real-time) and detection of PCR products, volume tested (200 μl—10 ml), DNA extraction procedures (mechanical versus enzymatic lysis of fungal cells), choice of target gene, and use of appropriate controls. All these factors contribute to the heterogeneity[30,31] and the less likelihood of PCR being accepted as the definitions of invasive fungal disease.[32] However, this heterogeneity can be reduced when the criteria to define a PCR test as positive is based on two consecutive samples being positive.[32] An improvement of establishing the standard for PCR could also be achieved by selecting two different genes, which can result in higher specificity and positive predictive value.[31,33]

In this study, unequivocal identification or repeat could not be achieved in two reference strains (*C. krusei* vs. *C. guilliermondii*), while other reference fungal strains used for assay development exhibited sufficiently distinguishable melting profiles with different T_m values. Evaluation of the newly developed method using 82 documented fungal isolates revealed accurate species identification of all specimens, and confirmed by 18S rRNA sequencing. The most important factors affecting the melting curve are the GC content and DNA-sequence length.[14,34] Here, we used an amplicon of appropriate size, since larger amplicons containing sequences with more different GC content allow better differentiation but less effective in detecting single-base substitutions. In clinical application, an unknown specimen not included in the reference library may possess the same Tm value as a non-associated reference strain, which possibly leads to a wrong interpretation. Therefore, our HRMA should be coupled with universal PCR and RFLP, which make the distinguish convincing.

For the 16 common fungal species of the developed universal PCR-RFLP assay, only 61.1% (11 out of 18) of fungal isolates from clinical samples can be detected at the species level. There were two reasons for this low sensitivity rate. First, residual samples after centrifugation and major specimens extracted for conventional cultures led to small pellet available for our PCR-HRMA assay, which limited the sensitivity. Second, the extraction method may not be able to destruct the mold and release its DNA as well, especially those of *Aspergillus* spp. and *Cryptococcus* spp.

Although this PCR-RFLP assay has been validated and proven useful, it has some limitations. The main problem of assessing the accuracy of this universal PCR-RFPT is no gold standard for diagnosis exists. A recent meta-analysis showed that the sensitivity of blood cultures in the diagnosis of invasive candidiasis is only 38%.[29] Therefore, it depends on an interventional study, most appropriately a

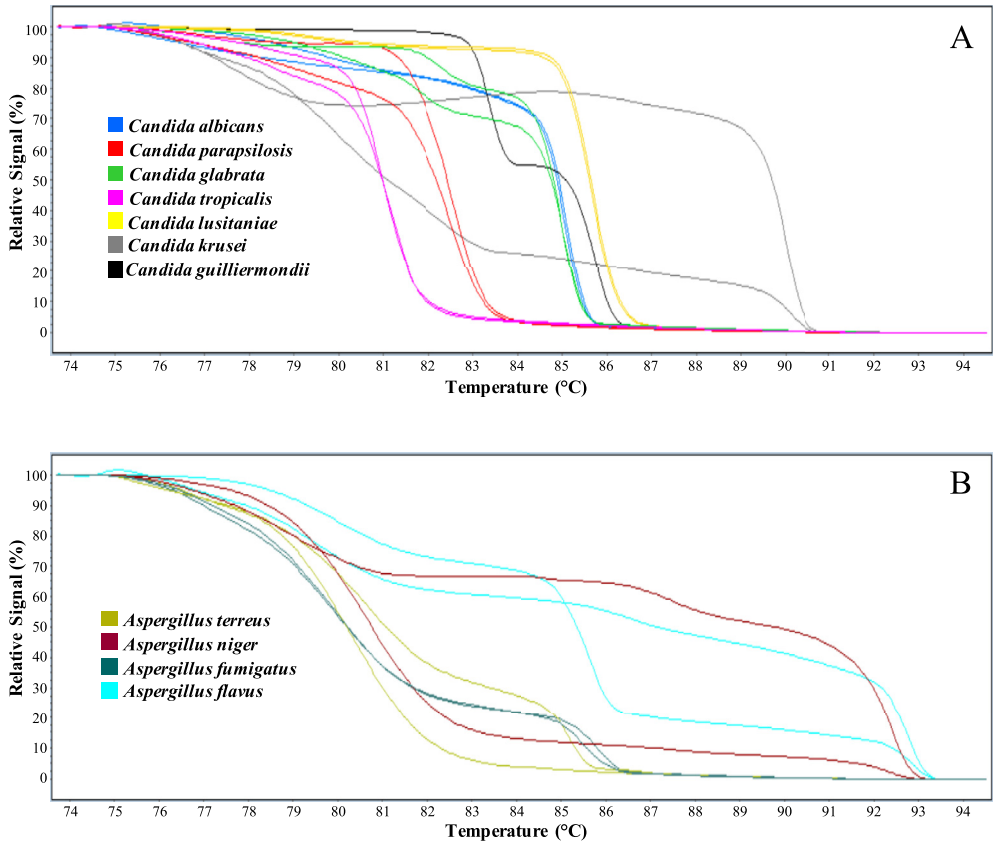


Figure 2. dMelt curves of reference fungal species in duplicates using high-resolution melting curve analysis.

Table 2 Performance of universal polymerase chain reaction (PCR) coupled with high-resolution melting analysis (HRMA) on the clinical samples from 75 patients.

Specimens types	Conventional culture identification results (no. of isolates)	Universal PCR-HRMA assay	Analysis result for discordant samples (based on sequencing)
Bronchoalveolar lavage (n = 45)			
Concordant samples (n = 33)	Negative (24), *C. albicans* (1), *C. glabrata* (4), *C. tropicalis* (1) Yeast-likes (3)	Negative (24), *C. albicans* (1), *C. glabrata* (4), *C. tropicalis* (1) *C. albicans* (1), *C. glabrata* (1) and *C. guilliermondii* (1)	The same as universal PCR-HRMA assay
Discordant samples (n = 12)			
Of the 16 fungi species	*A. fumigatus* (1), *A. flavus* (1) and *Crytococcus neoformans* (1)	Negative (3)	
Other uncommon fungi	*A. versicolar* (1), *Penicillium* sp. (2), *Acremonium* (2),	Negative (5)	
	Mold (4) (unidentified)	Negative (4)	Unidentified
Pleural fluids (n = 17)			
Concordant samples (n = 16)	Negative (14) *C. albicans* (1), *C. parapsilosis* (1)	Negative (14) *C. albicans* (1), *C. parapsilosis* (1)	
Discordant sample (n = 1)	*C. parapsilosis* (1)	Negative (1)	
Cerebrospinal fluid (n = 12)			
Concordant samples (n = 9)	Negative (9)	Negative (9)	
Discordant sample (n = 3)	*C. albicans* (1), *Cryptococcus* spp. (1), *C. neoformans* (1)	Negative (3)	
Synovial fluids (n = 1)			
Concordant samples (n = 1)	Negative (1)	Negative (1)	

randomized controlled trial, investigating whether management directed by this PCR-based tool improves patients' outcomes. Given the lack of antifungal susceptibility information from this PCR-RFLP assay, it can be applicable only as an adjuvant, rather replace the standard culture methods. Furthermore, it is difficult to interpret polymicrobial infections and samples with high levels of contaminating DNA sequences using this universal PCR assay.

In conclusion, our universal PCR-RFLP is a simple, efficient and rapid alternative of identifying invasive fungal species from clinical specimens of high risk patients. We suggest testing of patients with suspected invasive fungal disease by this diagnostic combination and accompany, instead of replacing blood cultures, and serial sampling should be considered. A consensus on the standardization of universal PCR assay, along with validation from large prospective studies, is necessary to allow widespread adoption of this PCR-RFLP assay. Future study is warranted to investigate whether the PCR-based fungal diagnostic assays guide antifungal therapy can lower patient mortality and decrease unnecessary antifungal treatment.

Ethics approval and consent to participate

The study was approved by the Institutional Review Board and Human Research Ethics Committee of CGMH with informed consent obtained from all patients.

Consent to publish

This study has not been published elsewhere, was not considered by any other journal, and all authors agreed to publish this study if accepted.

Availability of data and material

All information, data and material are presented within the manuscript.

Competing interests

All authors declare no conflicts of interest in this article.

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Authorship/contribution

Ming-Horng Tsai: Dr. Tsai conceptualized and designed the study, drafted the initial manuscript, and approved the final manuscript as submitted.

Lee-Chung Lin: Dr. Lin performed the microbiological characteristics of this study, help the laboratory analysis and initial manuscript writing.

Jen-Fu Hsu: Dr. Hsu designed the data collection instruments, and coordinated and supervised data collection and the whole study.

Mei-Yin Lai: Dr. Lai performed the antifungal testing of this study, and helped to collect and verify the data.

Hsuan-Rong Huang: Dr. Huang helped to perform the statistical analysis.

Ming-Chou Chiang: Dr. Chiang took care of these patients, and helped data verification.

Jang-Jih Lu: Dr. Lu critically reviewed the manuscript, revised the manuscript, and approved the final manuscript as submitted.

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