WALAILAK JOURNAL
http://wjst.wu.ac.th Life Sciences

Universal Multiplex Polymerase Chain Reaction-Restriction Fragment Length polymorphism (UMPCR-RFLP) for Rapid Detection and Species Identification of Fungal and Mycobacterial Pathogens[†]

Jidapa SZEKELY[1,*], Sureerat CHELAE[2], Natnicha INGVIYA[2], Weerapan RUKCHANG[2], Sauvarat AUEPEMKIATE[3] and Kumpol AIEMPANAKIT[4]

[1]*Faculty of Medical Technology, Prince of Songkla University, Songkhla 90110, Thailand*
[2]*Clinical Microbiology Unit, Faculty of Medicine, Prince of Songkla University, Songkhla 90110, Thailand*
[3]*Division of Anatomical Pathology, Department of Pathology, Faculty of Medicine, Prince of Songkla University, Songkhla 90110, Thailand*
[4]*Division of Dermatology, Department of Internal Medicine, Faculty of Medicine, Prince of Songkla University, Songkhla 90110, Thailand*

([*]Corresponding author's e-mail: jidapa.sz@psu.ac.th)

Received: 29 April 2019, Revised: 5 July 2019, Accepted: 19 August 2019

Abstract

Fungal and mycobacterial skin infections are common in immunocompromised patients and patients with febrile neutropenia, since the patients' ability to control localized infection is diminished by the disease. The similarity of the lesions caused by these organisms conduces to difficulty of differential diagnosis. Although a histopathological examination and a microbial culture are standard methods for laboratory diagnosis of skin infection, the methods have drawbacks. Histopathological examination yields low positive results, while microbial culture is time-consuming and might result in no growth, causing delayed treatment. This study aimed to develop and evaluate an in-house rapid polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for the detection and identification of fungal and mycobacterial pathogens in resource-limited laboratories. A total of 26 typed species of human pathogenic fungi and 12 species of mycobacteria were used. Strain differentiation was analyzed by using multiplex PCR-RFLP. The internal transcript spacer region (ITS) of fungi and heat-shock protein 65 (*hsp*65) gene of mycobacteria were amplified using ITS1-ITS4 and Tb11-Tb12 universal primers, respectively. The RFLP patterns were examined at genus-specific and species-specific level. No cross-amplification was observed between fungal and mycobacterial tested strains, nor any specific binding between primers and human DNA. It was concluded that the multiplex PCR-RFLP method developed in this study can be used as a molecular diagnostic test for fungal and mycobacterial species identification. In the future, this technique may be useful for detecting fungal and mycobacterial infection directly from clinical specimens.

Keywords: Multiplex PCR, PCR-RFLP, Fungal identification, Mycobacterial identification, Universal primers

[†]Presented at the International Conferences on Biomedical Sciences and Medical Technology 2019: June 20 - 21, 2019

Introduction

Fungal and mycobacterial skin infections are very common in immunocompromised patients and patients with febrile neutropenia. These patients are high-risk groups not only for local skin infection but also for disseminated infection, due to their defective host defense system. The systemic infection of these organisms is an important cause of morbidity and mortality among patients with neutropenia. The incidences of invasive fungal infection in patients with febrile neutropenia, including autopsied patients with hematological neoplasias, ranging from 10 to 21 %, depending on various factors, such as underlying disease and required treatment [1,2]. The incidence of non-tuberculous mycobacterial (NTM) infection in cancer patients with neutropenia is about 13 %, while in transplant patients, it can vary from 0.02 - 4.9 %, depending on the sites of organ transplantation [3,4].

The clinical presentation of skin infection with NTM or fungi is variable; hence it requires an accurately differential diagnosis. The subcutaneous nodules of cutaneous *Mycobacterium chelonae* lesions need to be differentiated from histoplasmosis, cryptococcosis, blastomycosis, coccidioidomycosis, sporotrichosis, actinomycosis and tuberculosis [5]. *M. marinum* infection can often be misdiagnosed as sporotrichosis due to similar clinical presentation as nodular lymphangitis [6]. Moreover, pulmonary tuberculosis is occasionally confused with invasive pulmonary aspergillosis in transplant recipients. The computed tomography findings of one-third of the pulmonary tuberculosis cases are very close to those of invasive pulmonary aspergillosis [7]. The consequence of misdiagnosis is long courses of antifungal therapy, which result in further spreading of the mycobacterium. Delayed diagnosis results in poor prognosis in immunocompromised patients and patients with febrile neutropenia.

Culture remains the gold standard for diagnosing mycobacterial and fungal infection. Culture-based methods have been phenotype driven, in which mycobacteria and yeasts were identified biochemically, and molds were identified based on morphological features [8,9]. These methods are still important, but they are limited by their turnaround time of several days to several weeks. Appropriate identification methods are needed for rapid and accurate diagnosis. Pathologists and clinicians are sometimes faced with the difficult situation whereby mycobacteria and fungi are identified by histologic methods, but speciation is not possible because no specimen was submitted for culture, the amount of specimen is low, or culture results are negative [10]. In an attempt to find a solution to this problem, PCR and other molecular methods in the detection and speciation of mycobacteria and fungi in biological samples are needed for routine diagnostic use.

Nowadays, molecular detection of fungal and mycobacterial infections in clinical specimens is available. Normally, the detection is performed separately by using different assays; however, none of the tests are designed to differentiate these infections at the same time. Fungal identification can be done by fungal internal transcribed spacer region or ribosomal small subunit (18S) rDNA PCR and amplicon sequencing [9,11]. Detection of mycobacterial infection is commercially available, such as Cobas Taqman MTB test and Xpert MTB/RIF assays [12,13]. However, these methods are expensive and require special equipment or service, which are not suitable for laboratories with limited resources available. Thus, the objective of this study was to design an in-house rapid, sensitive and specific molecular approach for the detection of fungal and mycobacterial DNA and for species identification. This paper focuses on a proof-of-concept UMPCR-RFLP by standardizing the technique. The method could determine fungal and mycobacterial DNA in the sample containing human DNA and could identify species of the organism. This method might be, further, applied for diagnostic purposes by using direct clinical specimens. The universal primers detecting ITS1-5.8S-ITS2 region of fungal rDNA and heat-shock protein 65 of mycobacterial DNA were used for multiplex PCR. The positive amplified products were subjected for species identification by restriction fragment length polymorphism and direct sequencing.

Materials and methods

Sample collection

A total of 26 species of common causative agents of subcutaneous fungal infection and 12 species of mycobacteria used in this study are listed in **Tables 1** and **2**. All 54 clinical isolates were collected from the Department of Clinical Microbiology in Songklanagarind Hospital, Hat Yai, Thailand, from Jan. 2016 to Jan. 2017. This study was conducted according to the principles expressed in the Declaration of Helsinki. All protocols were approved by the Ethics Committee of the Faculty of Medicine, Prince of Songkla University (ethical number: REC58-153-19-2). The cultures were isolated from clinical specimens collected from sterile sites without normal flora such as blood, tissue and body fluid. The basic species identification of all fungal and mycobacterial isolates was performed following the criteria of routine laboratory procedures at Songklanagarind Hospital. Briefly, molds were identified by morphology identification, yeasts were identified by sugar assimilation test, and mycobacteria were identified by growth rate, colony morphology and biochemical tests. The fungal cultures were grown on Sabouraud dextrose agar at 30 °C, ambient air condition. The mycobacterium isolates were cultured on Löwenstein-Jensen medium at 37 °C for 3 - 5 days with the rapid-growing mycobacterium and 4 weeks with the slow-growing species.

Fungal genomic DNA extraction

Mycelium DNA was extracted using boiling technique. Briefly, 2 - 5 day old cultures were suspended in 700 µL of extraction buffer [2 % Triton X-100, 1 %SDS, 100 mM NaCl, 10 mM Tris-Cl buffer (pH 8.0), 1 mM EDTA] and boiled for 15 min. Cell debris and proteins were removed with 500 µL of phenol/chloroform. Genomic DNA was recovered using ethanol precipitation. The DNA pellet was resuspended in 50 µL of 10 mM Tris-Cl (pH 8.0). DNA concentration and the purity were measured by UV-Vis spectrophotometer (NanoDrop 2000, Thermo Scientific). A final concentration of 100 ng/µL of DNA samples was prepared for further use.

Mycobacterium genomic DNA extraction

Mycobacterium colonies were grown on Lowenstein-Jensen media. One loop full of colonies was suspended in 1 mL of Tris-EDTA (10mM Tris, 1mM EDTA, pH 8.0) and then heat-inactivated at 80 °C for 10 min. Cell debris was removed by centrifugation at 12,000 rpm for 15 min. The supernatant was collected and frozen at -20 °C until use.

Simplex PCR amplification and sequencing

Species of all isolates used in this study were confirmed by sequencing. The internal transcribed spacer (ITS) regions of fungi and *hsp*65 gene of mycobacterium were amplified using 2 primer sets ITS1/ITS4 [14] and Tb11/Tb12 [15], respectively. PCR was performed in a 50-µL volume consisting of 25 µL TopTaq Master mix kit (Qiagen, Germany), 20 pmol of each primer, and 100 ng of DNA template. The positive controls consisted of 100 ng of DNA *Talaromyces marneffei* and *Mycobacterium tuberculosis* strain H37Rv. The negative control consisted of 10 mM Tris-Cl buffer (pH 8.0) without the DNA template. The amplification protocol was performed as follows: initial denaturation at 94 °C for 3 min; 35 cycles at 94 °C for 45 s, 55 °C for 30 s, 72 °C for 30 s; and a final extension at 72 °C for 10 min. PCR amplification was carried out on Thermal cycler (BioRad®). The samples were run in 2 % agarose and visualized using ethidium bromide.

Sequencing analysis

The ITS region and *hsp*65 gene of all clinical isolates were amplified and sequenced from both sides. All sequencing processes were serviced by 1st BASE DNA Sequencing (Apical Scientific, Malaysia). The sequences were analyzed and manually edited using MEGA program version X. Species of the organisms were determined by BLASTn comparisons against GenBank sequences.

Optimal annealing temperature of Universal-Multiplex PCR assay

To allow rapid detection and differentiation between fungal and mycobacterium infection, clear visualization of causative agent's DNA by gel electrophoresis and fast result are needed for accurate treatment. The conventional multiplex PCR technique was chosen, due of its simplicity and low requirement of complicated and expensive instrumentation, which allow this technique to be performed in all laboratories with limited resources. The universal Tb11/Tb12 primers for detection of heat-shock protein 65 gene of mycobacterium (440 bp) and the universal ITS1/ITS4 primers detecting internal transcribed spacer region of fungal DNA (500 - 900 bp) were selected in order to obtain clear sequential amplicons separating between mycobacterial and fungal DNA. To allow an equal amplification of both targets, the optimal annealing temperature was determined by performing PCR using annealing temperatures of 50 to 65 °C. Multiplex PCR was performed with 2.5U TopTaq DNA polymerase (Qiagen, Germany) in 50-µL reactions. PCR reactions contained 1x TopTaq PCR buffer, 1.5 mM $MgCl_2$, 0.6 mM each of dATP, dTTP, dCTP and dGTP, 10µM of each primer and 100 ng of each DNA sample. The amplification was performed in a BioRad® thermocycler, with an initial denaturation at 94 °C for 3 min; 35 cycles at 94 °C for 40 s, 50 to 65 °C for 30 s, 72 °C for 60 s; and a final extension at 72 °C for 10 min. The samples were run in 2 % agarose and visualized using ethidium bromide.

Sensitivity of multiplex PCR assay

The sensitivity of multiplex PCR amplification was determined on purified *Talaromyces marneffei* and *Mycobacterium tuberculosis* DNA. DNA concentration was measured using NanoDrop spectrophotometer (Thermo Scientific™). Tenfold serial dilutions of the purified DNA samples varying from 100 ng to 1 fg were prepared with molecular graded water as a diluent and then subjected to multiplex PCR at the optimized condition.

Specificity of the multiplex PCR assay

Specificity of the ITS1/ITS4 primers was tested with mycobacterial DNA of *M. abscessus, M. fortuitum,* and *M. tuberculosis*. Specificity of Tb11/Tb12 primers was determined with *Aspergillus fumigatus, Penicillium citrinum,* and *Cryptococcus neoformans.* Non-specific binding of both primer pairs was tested with serial dilutions of human DNA. All specificity tests were performed at the optimized multiplex PCR condition.

Species analysis by PCR-RFLP

Amplified PCR products of fungal DNA were digested with *Mbo*II, *Hae*III and *Hha*I restriction endonuclease enzymes (Thermo Scientific™). The mycobacterial DNA amplicons were analyzed using *Bst*EII and *Hae*III enzyme (Thermo Scientific™). The restriction fragments were separated on a 3 % (wt/vol) agarose gel followed by staining in ethidium bromide and visualized UV transillumination. Lengths of DNA fragments were determined with standard DNA marker using UVITEC software (Cambridge, United Kingdom). The RFLP patterns from reference sequences were determined using NEBCUTTER ver. 2.0 and the online database app.chuv.ch/prasite/index.html [15].

Results and discussion

Multiplex PCR optimal annealing temperature

The optimal annealing temperature for multiplex PCR assay was determined by performing PCR with annealing temperature ranging from 50 to 65 °C. The optimal annealing temperature for both ITS1/ITS4 and Tb11/Tb12 primer pairs was not significantly different, ranging from 50 to 60 °C (**Figures 1A** and **1B**). Intensity of amplicons were seen clearly for both PCR assays. The 59 °C annealing temperature was a balance between the annealing temperatures of the ITS and *hsp*65 PCR assays.

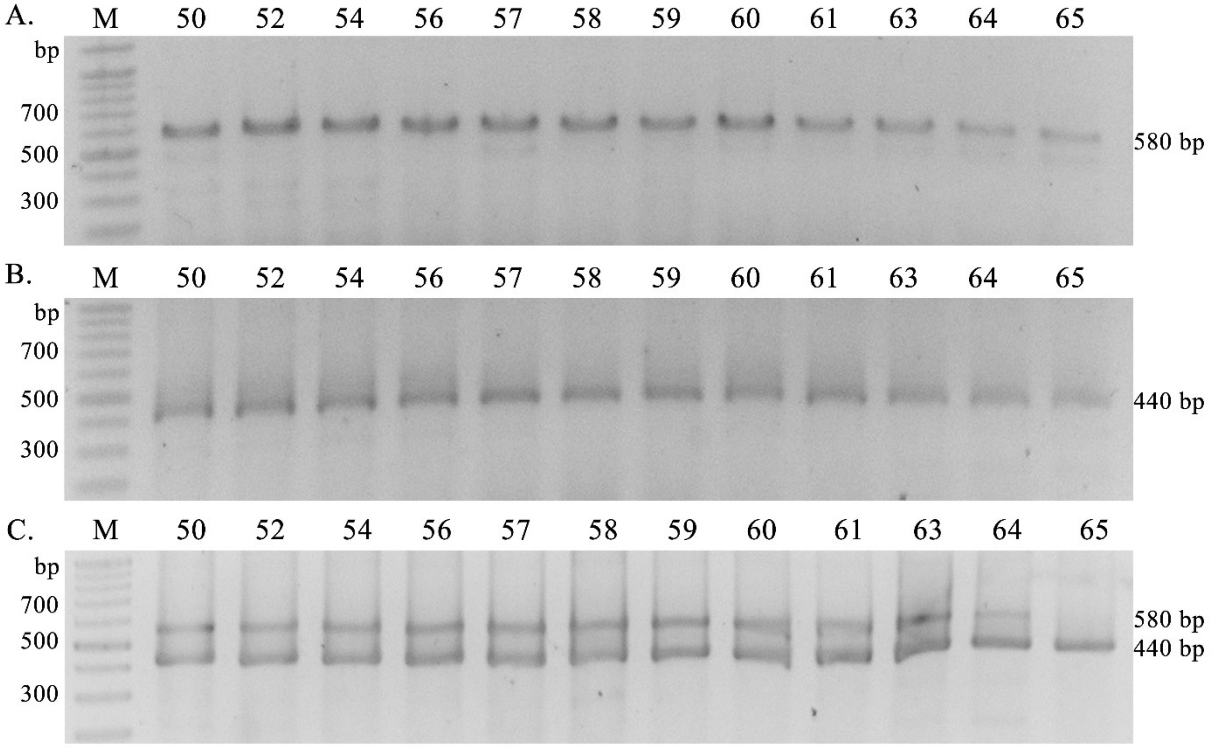

Figure 1 The effect of annealing temperature on PCR amplification. Panel A is a multiplex PCR mixture containing *Talaromyces marneffei* DNA template showing a 580-bp amplified PCR product. Panel B is a multiplex PCR mixture containing *Mycobacterium tuberculosis* DNA template showing a 440-bp amplicon. Panel C is a multiplex PCR mixture containing both *T. marneffei* and *M. tuberculosis* DNA templates. Lane M shows 100-bp DNA ladder; lanes 1 - 12 show annealing temperatures ranging from 50 to 65 °C

Sensitivity of multiplex PCR assays

The sensitivity of the multiplex PCR assay was found to be lower than the individual PCR, as shown in **Figure 2**. The ITS region of fungal DNA could be detected at an amount as low as 100 pg of DNA, while the *hsp*65 gene of mycobacterial DNA could be detected at an even lower amount of 10 pg of DNA. The multiplex PCR assay showed a detection sensitivity of 1 ng of fungal DNA and 100 pg of mycobacterial DNA. This could indicate a possible correlation between shorter PCR products and higher detection limit of the primers. A previous study showed the sensitivity for the single amplification of the ITS/5.8S rDNA region to be 1 fg for *C. albicans* and 10 fg for *A. fumigatus* DNA, which corresponds to about 1 to 10 cells of *C. albicans* and fewer than 100 cells of *A. fumigatus,* respectively [16]. The limited sensitivity of Tb11/Tb12 primers for *hsp65* gene of mycobacterium was about 100 pg (about 3.5 ± 2.5 $\times 10^3$ CFU/mL) for *M. tuberculosis* [17]. Our results disclosed a similar detection limit of *hsp65* gene for mycobacterium, however, the sensitivity for fungal ITS region detection was lower than that in the previous study. This demonstrates that the multiplex PCR tends to favor smaller PCR product fragments.

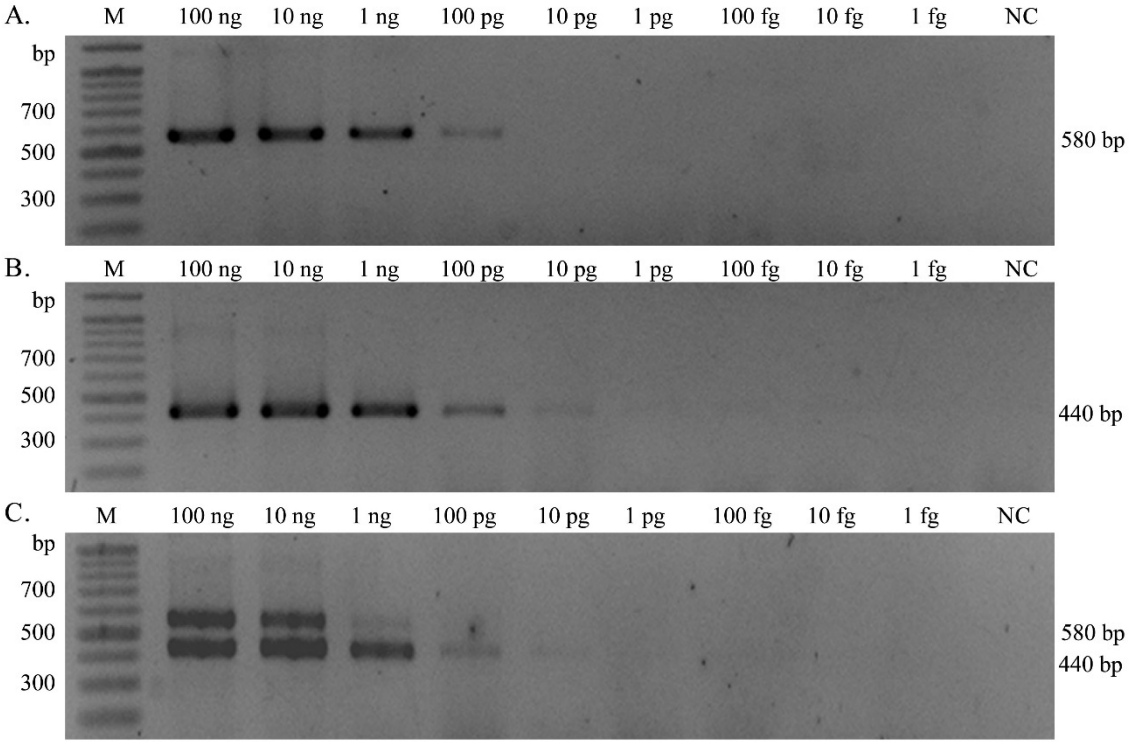

Figure 2 Sensitivity of multiplex PCR in detection of ITS region of *Talaromyces marneffei* and *Hsp*65 gene of *Mycobacterium tuberculosis*. Panel A is a multiplex PCR mixture containing *T. marneffei* DNA template. Panel B is a multiplex PCR mixture containing *M. tuberculosis* DNA template. Panel C is a multiplex PCR mixture containing both *T. marneffei* and *M. tuberculosis* DNA templates. Lane M shows 100-bp DNA ladder; lanes 1 - 12 are concentrations of each DNA template varying from 100 ng to 1 fg; lane NC is negative control without DNA template.

Specificity of multiplex PCR assays

The ITS1/ITS4 primer pairs showed no amplified product with DNA samples from mycobacteria. The Tb11/Tb12 primer pairs were also specific to mycobacterial DNA but not to fungal DNA samples (data not shown). None of the primer pairs yielded products with tested human skin DNA sample. The multiplex PCR amplified expected PCR products of *A. fumigatus, P. citrinum, T. marneffei, M. abscessus, M. fortuitum, M. tuberculosis* (**Figures 3A** and **3B**). No non-specific amplification of the multiplex PCR assays was shown, when tested with 100 ng, 10 ng and 1 ng of human DNA samples (**Figure 3C**). These results showed no cross-reactivity between fungal and mycobacterial pathogens or human DNA sequences.

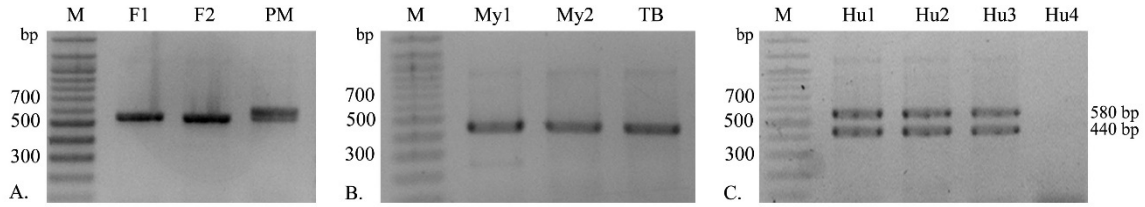

Figure 3 Specificity of multiplex PCR. Panel A shows products amplified from DNA of *Aspergillus fumigatus* (F1)*, Penicillium citrinum* (F2)*,* and *Talaromyces marneffei* (PM) mixed with 100 ng of human DNA. Panel B shows amplicons of *Mycobacterium abscessus* (My1)*, M. fortuitum* (My2) and *M. tuberculosis* (TB) DNA mixed with 100 ng of human DNA. Panel C shows amplified products of *T. marneffei* and *M. tuberculosis* DNA samples mixed with 100 ng (Hu1), 10 ng (Hu2) and 1 ng (Hu3) of human DNA; lane Hu4 is a multiplex PCR mixture containing 100 ng of human DNA.

Species identification by Restriction Fragment Length Polymorphisms (RFLP)

The fungal and mycobacterial species identification by universal PCR-RFLP was confirmed by sequencing of the ITS region and *hsp*65 gene PCR amplicons, and comparison with sequences from the GenBank database.

Amplified PCR products of multiplex PCR containing universal primers for fungal ITS regions and mycobacterial *hsp65* genes were directly subjected to restriction endonuclease enzyme digestion**.** Lengths of PCR products amplified from all tested fungal DNA samples ranged between 500 - 920 bp, while the products from all mycobacterial DNA used in this study were 440 bp.

RFLP patterns for fungal species identification were predicted according to the sequences obtained from the GenBank database. Restriction patterns of fungal ITS amplicons treated with *Mbo*II, *Hae*III and *Hha*I restriction enzymes were estimated using NEBcutter v.2.0 and tested on fungal isolates listed in **Table 1** and **Figure 4**. The RFLP patterns of mycobacterial *hsp*65 gene amplicons were obtained by *BstE*II and *Hae*III endonuclease enzyme treatment. Agarose gel electrophoresis was used for determination of fragment sizes, due to its convenient and easy setup for routine work in clinical laboratories. The 10-bp fragment size difference was detectable, and the characteristic RFLP patterns were reliable and repeatable. The RFLP patterns of all tested fungal and mycobacterial strains and the predicted ones of reference sequences were concordant. For fungal identification, we found that *Mbo*II pattern was species specific, while *HaeIII* and *HhaI* patterns were found similar within genus such as *Aspergillus* and *Candida*. To save time and cost, 2 restriction enzymes either *MboII-HaeIII* or *MboII-HhaI* can be used for fungal species identification.

Thirty-eight isolates of the medically important human pathogenic fungi and 17 isolates of *M. tuberculosis* and Non-tuberculous mycobacteria (NTM) were tested in this study (**Tables 1** and **2**). For fungal species identification, *Mbo*II patterns demonstrated species specific while *Hae*III and *Hha*I patterns showed genus specific. RFLP patterns of ITS region using 2 endonuclease enzyme treatment (*Mbo*II with either *Hae*III or *Hha*I), were specific enough for accurate identification (**Table1** and **Figure 4**). For mycobacterium identification, the use of both *BstE*II and *Hae*III endonuclease was necessary (**Table 2** and **Figure 5**).

To ensure the performance of the test, all RFLP patterns of the tested isolates were confirmed by determining the restriction sites of the sequenced ITS and *hsp65* gene amplicons using NEBcutter v.2.0. The reproducibility of the RFLP assay was determined by performing the assay 3 times by 3 different researchers, with similar results obtained. Some important mycobacterial species causing skin infection could not be obtained for our study; the RFLP patterns of such species were previously studied and available in the online database app.chuv.ch/prasite/index.html [15]. No RFLP pattern repetitions were found among the 35 tested species, indicating 100 % analytical specificity for species determination.

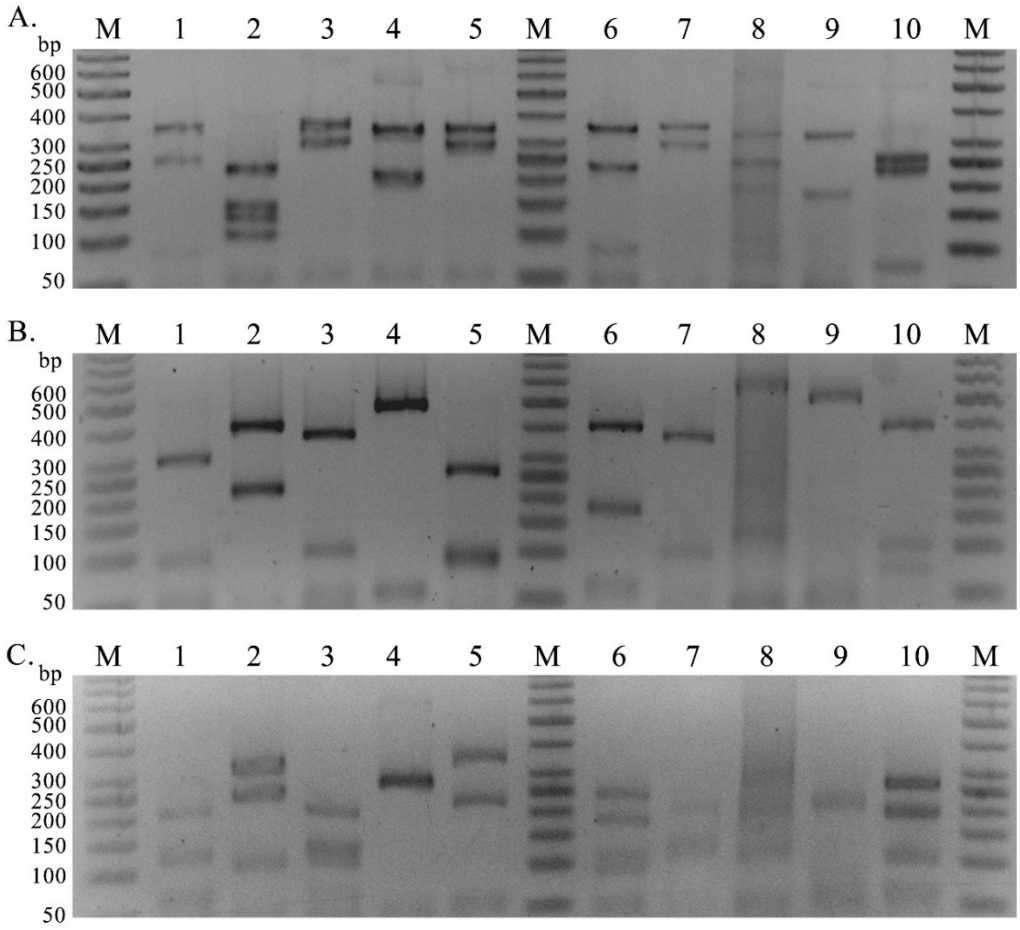

Figure 4 RFLP patterns of fungal ITS region treated with *Mbo*II (A), *Hae*III (B) and *Hha*I (C). Lane M = 50-bp DNA ladder marker; lane 1 = *Trichosporon rubrum*; lane 2 = *Rhodotorula*; lane 3 = *Microsporum gypseum*1; lane 4 = *Geotrichum candidum*; lane 5 = *Histoplasma capsulatum*; lane 6 = *Fonseceae pedrosoi*; lane 7 = *Microsporum gypseum*2; lane 8 = *Aspergillus niger*; lane 9 = *Candida krusei*; lane10 = *Talaromyces marneffei*.

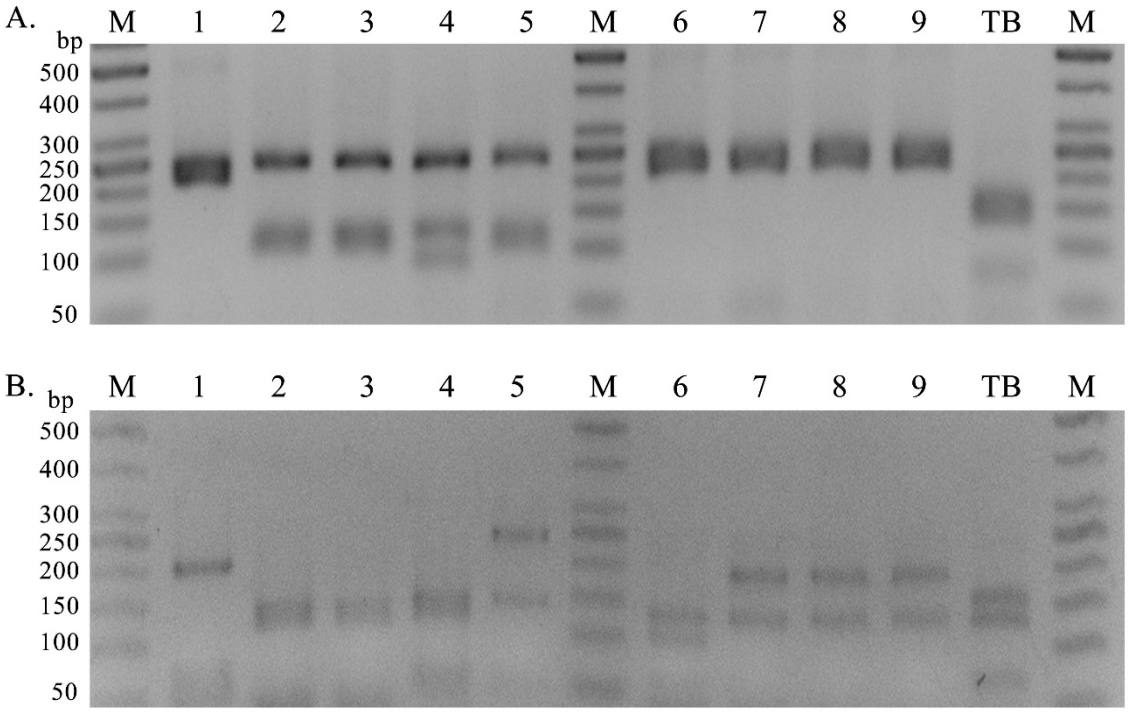

Figure 5 RFLP patterns of mycobacterial *Hsp*65 gene amplicons treated with *Bst*EII (A), *Hae*III (B). Lane M = 50-bp DNA ladder marker; lane 1 = *Mycobacterium abscessus* M4903-01; lane 2 = *M. gordonae* M426-01; lane 3 = *M. intracellulare* M3390-12; lane 4 = *M. fortuitum* M5067-12; lane 5 = *Mycobacterium* sp. M3243-2, ; lane 6 = *M. kubicae* M3721-11; lane 7 = *M. genavense* M152-01; lane 8 = *M. genavense* M2033-1; lane 9 = *M. genavense* M2168-01, Lane TB = *M. tuberculosis* positive control

Table 1 Length and restriction fragment length polymorphism pattern of the amplified ITS1-5.8s-ITS2 regions in tested fungal isolates.

Organism	GenBank Access. no.	Amplicon length (bp)[a]	*Mbo*II fragments (bp)[b]	*Hae*III fragments (bp)[b]	*Hha*I fragments (bp)[b]	Tested isolates
Fungi						
Aspergillus flavus	MG991638	600	330/250	300/100/70	190/140/90	F1669, AF0001
Aspergillus fumigatus	KX349486	600	330/150/90	270/100/70	190/130/90	F2765, F3789, F4910
Aspergillus niger	MH266204	580	320/250/190	580	285/200/120	F1209
Cladosporium endophytica	NR158360	547	225/150	420/70/50	300/150/90	F4626
Cladosporium oxysporum	MF326607	760	345/230/80	415/180	240/180/110/95	F4928
Curvularia verruculosa	KP698724	620	350/200	400/100/80	220/150/100	CV0001
Fusarium fujikuroi	KJ000432	550	300/225	340/110/90	300/260	F2021
Fusarium solani	KX385049	600	250/150/90	260/120/100	300/200/100	FS0001
Histoplasma capsulatum	KP132276	670	350/290	270/90	350/225	HC0001
Penicillium citrinum	KT844552	600	350/230	260/110/80/20	200/100	PC0001
Pythium insidiosum	EF016852	920	450/320/150	500/110	680/280/100	CBS119452
Scedosporium apiospermum	AY213683	685	230/150/120/65	420/100	220/90	PD2014
Scedosporium aurantiacum	KP132679	700	250/210/180	290	600	F2437
Sporothrix schenckii	NR_147566.1	525	300/225	275/110/80	220/70/60	SS0001
Talaromyces marneffei	KP780451	580	260/240/70	400/100/80	260/170/100/50	TM0001, F3080
Trichophyton rubrum	NR131330	740	365/260/80	310/100	220/135	TR0001
Microsporum gypseum	KX668866	720	355/290	380/95	210/125	MG0001
Yeasts						
Candida albicans	KU729037	540	240/220/70	450/90	280/260	F1894, F4869, F340
Candida glabrata	KP675601	900	500/400	680/220	400/150/140	CG0001
Candida krusei	LC389030	500	300/170	350/90	200/180/70	F3457
Candida guilliermondii	MK267756	635	390/200/80	400/120/85	330/280	CP22019
Candida parapsilosis	LC390149.1	515	220/215/80	400/100	290/225	CP216-06, CP4632-12
Candida tropicalis	KY102464	540	310/230	450/85	280/260	CT2056, CT1592, CT3991
Cryptococcus neoformans	MF490470.1	557	255/200/100	455/105	285/265	F291, F2727, F2067
Rhodotorula mucilaginosa	KF646193	620	230/150/130/100	400/220	300/230/100	RM2015
Trichosporon asahii	MF662389.1	550	340/210	490	270	F1961, F1545

[a]Lengths of amplicons calculated from GenBank
[b]Lengths of amplicon fragments after digested with endonuclease *Mbo*II, *Hae*III and *Hha*I

Table 2 Length and restriction fragment length polymorphism pattern of the amplified heat-shock protein 65 gene in tested mycobacterium isolates.

Organism	GenBank Access. no.	Amplicon length (bp)	*Bst*EII fragments (bp)	*Hae*III fragments (bp)	Tested isolates
Mycobacterium abscessus	DQ869273	440	235/210	200/70/60	M4903-01, M406-02
Mycobacterium asiaticum	AY299133	440	235/210	115/105	M218-12
Mycobacterium avium s. avium	DQ284768	440	235/210	130/105	M2691-12
Mycobacterium brisbanense	AY943196	440	235/120/100	140/125/100	M5065-12
Mycobacterium fortuitum	AY299152	440	235/120/85	145/120/60	M5067-12, M2569-2, M5293-2
Mycobacterium genavense	AY299183	440	235/210	185/130	M2168-01, M152-01, M2033-1
Mycobacterium gordonae	AJ310238	440	235/120/100	130/115	M426-01, M3390-12, M4002-12
Mycobacterium intracellulare	AF126035	440	235/120/100	145/130/60	ND[c]
Mycobacterium kubicae	AY373458	440	235/210	140/100/60	M3721-11
Mycobacterium tuberculosis complex	AY299144	440	235/120/85	150/130/70	H37Rv, M3558-2
Mycobacterium chelonae	AY299148	440	320/130	200/60/55	ND[c]
Mycobacterium marinum	AY299134	440	235/210	145/105/80	ND[c]

[a]Lengths of amplicons calculated from GenBank
[b]Lengths of amplicon fragments after digested with endonuclease *Bst*EII and *Hae*III,
[c]Not done in this study; data of RFLP pattern from Telenti *et al*., 1993 [15]

Several causative agents of fungal and mycobacterial infection, especially in febrile neutropenic (FN) patients and immunocompromised patients, are reported worldwide. Febrile neutropenia is a medical emergency with high mortality without appropriate treatment. Fungal pathogens are common in high-risk patients. with *Candida* spp. and *Aspergillus* spp. being responsible for the most invasive fungal infections during neutropenia [18]. *Histoplasma* sp. and opportunist fungi such as *Fusarium* spp., along with atypical mycobacteria, are a cause of concern for FN patients with skin infections [19]. For immunosuppressed patients, *Mycobacterium avium* complex is the most common cause of NTM infection of all sites of infection, while rapid growers, including *M. abscessus, M. chelonae*, and *M. fortuitum*, play an important role in skin infection [20].

The proposed technique, UMPCR-RFLP, could be, further, used as a differential diagnostic tool for fungal and mycobacterial infection in clinical specimens. This method preliminarily resulted in very rapid turnaround time, totally about 8 h, for the detection of fungal and mycobacterial pathogens. Early results for the presence or absence of fungal or mycobacterial DNA in the sample were achieved within 4 h, with an additional 4 h necessary for the species identifications. In comparison, the general turnaround time of the default routine identification process was at least 3 - 14 days for mold, 48 - 72 h for yeast and 4 - 8 weeks for mycobacteria. The most time-consuming aspect was found to be the establishment of a pure culture of a suspected etiological organism isolated from clinical samples. Rapidly receiving a positive or negative test result may assist the treating physicians in their decision to continue or stop prophylactic therapy that can have severe adverse effects (e.g. hepatoxicity and nephrotoxicity of antifungal drug) for the patient [21]. However, an algorithm of our UMPCR-RFLP for fast and reliable detection and identification in terms of clinical use still needs to be evaluated further by using a set of well-characterized clinical samples.

The PCR-RFLP technique is still widely used for species identification of fungi. Dermatophyte species (*Epidermophyton* spp., *Microsporum* spp. and *Trichophyton* spp.) related to dermatophytosis were identified using PCR products of ITS1-5.8S-ITS2 region of rDNA treated with *Mva*I restriction enzyme [22,23]. The process of species identification by PCR-RFLP with *Mva*I was completed within 5 h. For our study, all processes of identification could be completed within 8 h. We suggest the processes to be shortened by using commercial DNA extraction kits and restriction enzymes with fast-digest activity. Moreover, the species identification of medically important yeasts and dimorphic fungi using PCR-RFLP with NL4-ITS5 primers and *Dde*I digestion can be performed with biological materials from a variety of sources such as blood, CSF and bone marrow [24]. The studies have proven that the DNA-based assays targeting the rDNA ITS regions provide reliable screening tools for species identification and for epidemiological studies. Some drawbacks of the PCR-RFLP method need to be closely examined. Dermatophyte species studies showed 2 ITS-*Mva*I RFLP distinctive patterns for *T. interdigitale* and *M. gypseum* [22]. In this case, the ITS-sequencing should be used for species confirmation. Moreover, to identify closely related species such as *Candida albicans* and *C. dubliniensis*, an additional endonuclease enzyme treatment was necessary [25].

A PCR-RFLP method for mycobacterial identification has been developed, with heat-shock protein65 (*hsp*65) and RNA polymerase (*rpo*B) gene being used as a target for PCR amplification [15,26]. The evaluation of these 2 target genes was studied by Ong *et al.* [27], resulting in the conclusion that the PCR-RFLP of the *hsp*65 gene is more useful than that of the *rpoB* gene in terms of accuracy of species identification.

Conclusions

This study has developed a novel universal multiplex PCR-RFLP assay for the detection and identification of human pathogenic fungi and mycobacteria. It combines the advantages of 2 sets of universal primers that are highly specific to the conserved regions of the target organisms, reliable and well-known for species identification, in order to create an effective tool for the differential detection between fungal and mycobacterial pathogens. This Universal multiplex PCR-RFLP technique also provides a rapid and reliable approach for 'broad-range' fungal and mycobacterial species identification. RFLP analysis of fungal strains newly-developed in this study and RFLP patterns of mycobacterial

species from a previous study provide a convenient and powerful method of species identification for laboratories with limited resources.

Acknowledgements

This work was supported by grant MET600082S from National Research Council of Thailand and Prince of Songkla University.

References

[1] J Auberger, C Lass-Flörl, H Ulmer, E Nogler-Semenitz, J Clausen, E Gunsilius, H Einsele, G Gastl and D Nachbaur. Significant alterations in the epidemiology and treatment outcome of invasive fungal infections in patients with hematological malignancies. *Int. J. Hematol.* 2008; **88**, 508-15.
[2] K Donhuijsen, P Petersen and WK Schmid. Trend reversal in the frequency of mycoses in hematological neoplasias: Autopsy results from 1976 to 2005. *Dtsch. Arztebl. Int*. 2008; **105**, 501-6.
[3] JM Gaviria, PJ Garcia, SM Garrido, L Corey and M Boeckh. Nontuberculous mycobacterial infections in hematopoietic stem cell transplant recipients: Characteristics of respiratory and catheter-related infections. *Biol. Blood Marrow Transplant* 2000; **6**, 361-9.
[4] BM Knoll. Update on nontuberculous mycobacterial infections in solid organ and hematopoietic stem cell transplant recipients. *Curr. Infect. Dis. Rep*. 2014; **16**, 421.
[5] P Chatzikokkinou, R Luzzati, K Sotiropoulos, A Katsambas and G Trevisan. Disseminated cutaneous infection with *Mycobacterium chelonae* in a renal transplant recipient. *Cutis*. 2015; **96**, E6-E9.
[6] JP Janik, RH Bang and CH Palmer. Case reports: Successful treatment of *Mycobacterium marinum* infection with minocycline after complication of disease by delayed diagnosis and systemic steroids. *J. Drugs Dermatol.* 2005; **4**, 621-4.
[7] SH Kim, MY Kim, SI Hong, J Jung, HJ Lee, SC Yun, SO Lee, SH Choi, YS Kim, JH Woo. Invasive pulmonary aspergillosis-mimicking tuberculosis. *Clin. Infect. Dis.* 2015; **61**, 9-17.
[8] C Mabilat, S Desvarenne, G Panteix, N Machabert, MH Bernillon, G Guardiola, P Cros. Routine identification of *Mycobacterium tuberculosis* complex isolates by automated hybridization. *J. Clin. Microbiol*. 1994; **32,** 2702-5.
[9] TR Kozel and B Wickes. Fungal diagnostics. *Cold Spring Harb. Perspect. Med*. 2014; **4**, a019299.
[10] S Mukhopadhyay and AA Gal. Granulomatous lung disease: An approach to the differential diagnosis. *Arch. Pathol. Lab. Med*. 2010; **134**, 667-90.
[11] K Wagner, B Springer, VP Pires, PM Keller. Molecular detection of fungal pathogens in clinical specimens by 18S rDNA high-throughput screening in comparison to ITS PCR and culture. *Sci. Rep*. 2018; **8,** 6964.
[12] SH Wang and P Pancholi. Mycobacterial skin and soft tissue infection. *Curr. Infect. Dis. Rep*. 2014; **16,** 438.
[13] X Qi. Diagnosis and therapy of rare central nervous system infection. *Neuroimmunol. Neuroinflammation*. 2014; **1**, 8-12.
[14] TJ White, T Bruns, S Lee, J Taylor. *Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics*. *In*: M Innis, D Gelfand, J Sninsky and T White (Eds.). PCR Protocols: A Guide to Methods and Applications. Academic Press, New York, 1990, p. 315-22.
[15] A Telenti, F Marchesi, M Balz, F Bally, EC Bottger and T Bodmer. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. *J. Clin. Microbiol*. 1993; **31**, 175-8.
[16] C Ferrer, F Colom, S Frasés, E Mulet, JL Abad and JL Alió. Detection and identification of fungal pathogens by PCR and by ITS2 and 5.8S ribosomal DNA typing in ocular infections. *J. Clin. Microbiol.* 2001; **39**, 2873-9.

[17] N Rastogi, KS Goh and M Berchel. Species-specific identification of *Mycobacterium leprae* by PCR-restriction fragment length polymorphism analysis of the *hsp65* gene. *J. Clin. Microbiol*. 1999; **37**, 2016-9.
[18] P Villafuerte-Gutierrez, L Villalon, J Losa, E Juan and C Henriquez-Camacho. Treatment of febrile neutropenia and prophylaxis in hematologic malignancies: A critical review and update. *Adv. Hematol*. 2014; **2014**, 986938.
[19] AV Mendes, R Sapolnik and N Mendonça. New guidelines for the clinical management of febrile neutropenia and sepsis in pediatric oncology patients. *J. Pediatr.* 2007; **83**, S54-63.
[20] E Henkle and KL Winthrop. Nontuberculous mycobacteria infections in immunosuppressed hosts. *Clin. Chest Med*. 2014; **36**, 91-9.
[21] S Girois, F Chapuis, E Decullier, B Revol. Adverse effects of antifungal therapies in invasive fungal infections: Review and meta-analysis. *Eur. J. Clin. Microbiol. Infect. Dis.* 2006; **25**, 138.
[22] R Mohammadi, M Abastabar, H Mirhendi, H Badali, S Shadzi, M Chadeganipour, P Pourfathi, N Jalalizand and I Haghani. Use of restriction fragment length polymorphism to rapidly identify dermatophyte species related to dermatophytosis. *Jundishapur J. Microbiol.* 2015; **8**, e17296.
[23] A Rezaei-Matehkolaei, K Makimura, SD Hoog, MR Shidfar, F Zaini, M Eshraghian, PA Naghan and H Mirhendi. Molecular epidemiology of dermatophytosis in Tehran, Iran, a clinical and microbial survey. *Med. Mycol*. 2013; **51**, 203-7.
[24] DRT Sousa, CSD Santos, B Wanke, RMDS Júnior, MCD Santos, KS Cruz, RL Monte, A Nocker, J Vicente and BD Souza. PCR-RFLP as a useful tool for diagnosis of invasive mycoses in a healthcare facility in the North of Brazil. *Electron. J. Biotechnol.* 2015; **18**, 231-5.
[25] M Dendis, R Horváth, J Michálek, F Růzicka, M Grijalva, M Bartos and J Benedík. PCR-RFLP detection and species identification of fungal pathogens in patients with febrile neutropenia. *Clin. Microbiol. Infect*. 2003; **9**, 1191-202.
[26] H Lee, HJ Park, SN Cho, GH Bai and SJ Kim. Species identification of mycobacteria by PCR-restriction fragment length polymorphism of the rpoB gene. *J. Clin. Microbiol*. 2000; **38**, 2966-71.
[27] CS Ong, YF Ngeow, SF Yap and ST Tay. Evaluation of PCR-RFLP analysis targeting *hsp65* and *rpoB* genes for the typing of mycobacterial isolates in Malaysia. *J. Med. Microbiol*. 2010; **59**, 1311-6.