Table 1. *Histoplasma capsulatum* isolates

Variety/	IFM no.	Strain name or number in other culture collection	Source	Country	Accession no.	Groups
capsulatum						
	5396	MTU 16001, TIMM 0713	Unknown	USA	AB176464	III
	5397	MTU 16002, TIMM 0714	Human/sputum	USA	AB176465	III
	5398	MTU 16003, TIMM 0715	Human/sputum	USA	AB176466	III
	5399	MTU 16010, TIMM 0722	Human/skin	USA	AB176467	III
	5400	MTU 16011, TIMM 0723	Unknown	USA	AB176468	III
	5401	MTU 16013, TIMM 0725	Human/lung	USA	AB176469	III
	5405	MTU 16018, IP 110575, TIMM 0730	Unknown	USA	AB176470	VI
	5407	MTU 16021, IP 637, TIMM 0733	Unknown	USA	AB176471	III
	5408	MTU 16031, B-580	Unknown	USA	AB176472	III
	5410	MTU 16033, B-17624	Unknown	USA	AB176473	IX
	5411	MTU 16034, B-2984	Unknown	USA	AB176474	III
	5412	MTU 16041, 80-4-3a	Unknown	USA	AB176475	III
	40752	12	Unknown	Unknown	AB176476	I
	41329	CDC 105	Human	USA	AB176477	IX
	41330	Ohino NHL 2966-1	Human	USA	AB176478	V
	41331	Ohino NHL 2966-2	Human	USA	AB176479	V
	46003	KUM 967, FMJ 502	Human	USA	AB176480	III
	46159	G 217B	Human	USA-Brazil*	AB176481	III
	47750	IBB	Human/lung	Japan-China*	AB176482	IV
	49200	CB-99-8	Human/skin	Brazil	AB176483	VI
	49721	Verdemi	Human/oral mucosa	Argentina	AB176484	VII
	50248	Benitez	Human/skin	Argentina	AB176485	VII
	50249	Fernandez	Human/oral mucosa	Argentina	AB176486	VII
	50250	Reggiani	Human/lymph node	Argentina	AB176487	VII
	50251	Gomez	Human/skin	Argentina	AB176488	VII
	50955	Myanmar	Human/lymph node	Japan-Myanmar*	AB176489	VII
	50958	NIH 02777	Human/skin	Thailand	AB176490	VII
	50959	NIH 02778	Human/blood	Thailand	AB176491	VII
duboisii						
	5406	MTU 16020, TIMM 0732, IP 634	Unknown	USA	AB176492	III
	5415	MTU 16022, IP 263	Unknown	Africa	AB176493	II
	5416	MTU 16023, IP 527, TIMM 0737	Unknown	Africa	AB176494	II
	5417	MTU 16024, TIMM 0738, IP 638	Unknown	Africa	AB176495	II
	41319	ATCC 22636, CBS 137.72(+)	Soil	USA	AB176496	III
	41320	ATCC 22635, CBS 136.72(−)	Soil	USA	AB176497	III
	41332	CDC B-650	Unknown	Africa	AB176498	II
	50954	Uganda	Human/CSF	Japan-Uganda*	AB176499	III
	52673	E2(2)	Human	Nigeria	AB176500	II
	52674	E7G	Human	Nigeria	AB176501	II
	52675	EB11	Human	Nigeria	AB176502	II
	52676	EB9	Human	Nigeria	AB176503	II
	52677	EGINT	Human	Nigeria	AB176504	II
farciminosum						
	5418	MTU 16042, 80-64-1a	Unknown	USA	AB176505	VII
	41333	SM 1024	Horse	USA	AB176506	VIII
	41335	CDC B-22	Horse	USA	AB176507	VIII
	41612	76103	Human/skin	Japan	AB176508	VII
	49109	Tsuchiura-1	Human/liver	Japan-Thailand*	AB176509	VII
	49110	Tsuchiura-2	Human/skin	Japan-Thailand*	AB176510	VII
	52876	SM 1025	Horse	USA	AB176511	II
	52877	SM 1026	Horse	USA	AB176512	VIII

Abbreviations for culture collections: ATCC, American Type Culture Collection, USA; CBS, Centraalbeaurau voor Schimmelcultures, The Netherlands; CDC, Centers for Disease Controls and Prevention, USA; IP, Institute Pasteur, France; KUM, Department of Dermatology, School of Medicine, Kanazawa University. Japan; MTU, Department of Bacteriology, Faculty of Medicine, University of Tokyo, Japan; NHL, National Institute of Hygienic Sciences, Japan; NIH, National Institute of Health Thai, Thailand; SM, Department of Dermatology, Shiga University of Medical Science, Japan; TIMM, Research Center for Medical Mycology, Teikyo University., Japan.; IFM, Institute of Food Microbiology, Chiba University; which was the former name of the Research Center for Pathogenic Fungi and Microbial Toxicoses.

*; isolated in Japan from patients of foreign nationality.

CSF, cerebrospinal fluid.

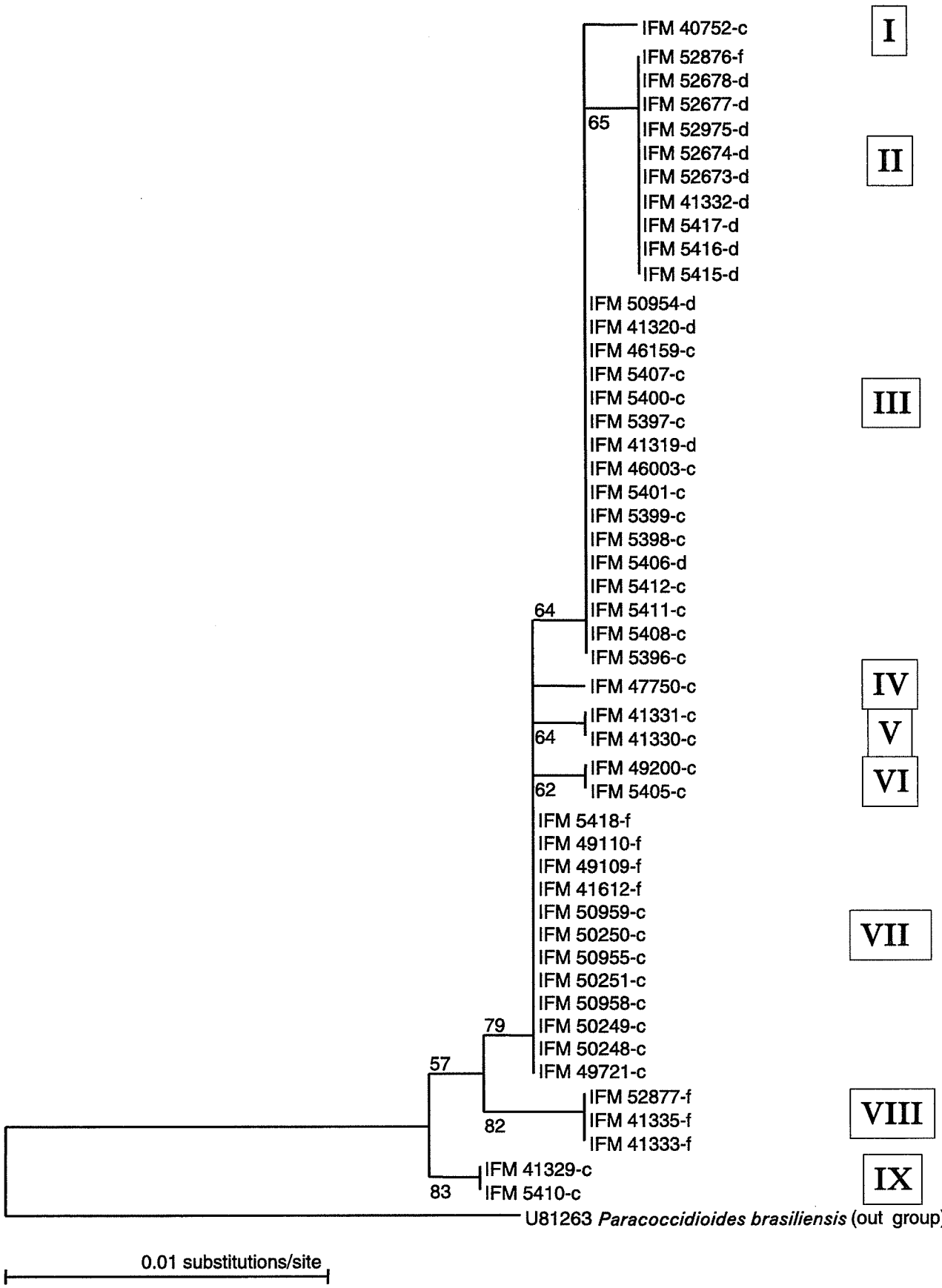

Fig. 1. Neighbor joining (NJ) tree for D1/D2 domains of three varieties of *Histoplasma capsulatum*. Bootstrap values derived from 10,000 replicates are shown as percentages.
The scale bar represents a difference corresponding to 0.01 (1.0%). "-c" indicates *H. capsulatum* var. *capsulatum*, "-d" incdicates *H. capsulatum* var. *duboisii*, and "-f" indicates *H. capsulatum* var. *farciminosum*

nt 1104 to 1130 of the D1/D2 region of the 28S rRNA gene of *Ajellomyces capsulatus* (Accession no. AF038354). The reaction mixture was subjected to 1 cycle of denaturation at 95°C for 4 min, 30 cycles of amplification at 94°C for 1 min, 55 or 58°C for 1 min, and 72°C for 2 min, and a final extension cycle at 72°C for 10 min with a PCR Thermal Cycler MP (TaKaRa). PCR products were separated by electrophoresis on 1.0% agarose gels in 1x TBE buffer (0.04 M Tris-boric acid, 0.001 M EDTA [pH 8.0]) and visualized by ethidium bromide staining. PCR products were purified with a PCR purification kit (QIAquick®, Qiagen, Tokyo, Japan), labeled with BigDye® terminator Ver. 1.1 (Applied Biosystems, Foster City, CA, USA) per the manufacturer's protocol and with primers of NL-1, NL-2[11], NL-2 Hc ver2, NL-3, and NL-4. The labeled samples were directly sequenced by ABI PRISM® 3100 sequencer (Applied Biosystems). DNA sequences were aligned with GENETEX-MAC genetic information processing software (Software Development Co., Ltd., Tokyo, Japan). Sequences were aligned with CLUSTAL W (version 1.6)[12]. A phylogenetic tree was constructed by the neighbor-joining (NJ) method. The sequence was listed accession number U81263 under Genbank, which is a sequence of a ribosomal RNA gene from *Paracoccidioides brasiliensis*, was used as an outgroup sequence for phylogenetic analysis. The nucleotide sequences for all strains examined were registered in the DNA Data Bank of Japan (DDBJ) under the accession numbers shown in Table 1.

Results and Discussion

Sequences generated as part of this study were entered into GenBank with serial accession numbers from AB176464 to AB176512 (Table 1). As shown in Fig. 1, the similarity values of the 49 isolates were 99.0% across the D1/D2 region of the 28S rRNA gene. These 49 isolates were then divided into 9 groups. None of the sequences were matched with the GenBank data such as AF038353, AF038354, AF071950, AF071951, AF071952, and AY235020 but was independent from the related sequences of *Onygenales*; *Ajellomyces dermatitidis* (AF038358), *Paracoccidioides brasiliensis* (U81263), and *Coccidioides immitis* (AY17613), and the similarity values were less than 96%. These results indicate that partial sequences of the D1/D2 region of the 28S rRNA gene are useful for species level identification of *H. capsulatum*.

Three of the groups: II, III and VII, consisted of 2 or 3 varieties that were confirmed by their site of isolation, host, cell size of parasitic form, internal transcribed spacer (ITS) regions of rRNA genes, and authorization by culture collections (Fig. 1). Some previous phylogenetic relationships of *H. capsulatum* isolates from a variety of regions established on the basis of DNA sequences of ITS regions of rRNA genes and partial sequences of genus encoding antigen precursor, fatty acid desaturase, alpha tubulin, ADP-ribosylation factor, H antigen precursor, and delta-9 fatty acid desaturase were questionable[3-5]. The isolates belonging to the genotypes consisting of different varieties had originated from distant countries. The results that the 9 groups were independent of the 3 varieties, reinforce these suspicions.

Acknowledgements

This study was performed as a part of the "Frontier Studies and International Networking of Genetic Resources in Pathogenic Fungi and Actinomycetes (FNGRPF)" through Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology of Japan (grant No. 10307006) and by the Japanese Ministry of Health, Labor and Welfare (grant No. H15-Special-06 and 15091901). Takashi Komori would like to thank Dr. Toshihiro Okajima at Fujisawa Pharmaceutical Research and Development Labs, Astellas Pharma Inc., for providing the opportunity to pursue this study.

References

1) Kwon-Chung KJ, Bennett JE: 18. Histoplasmosis. *In* Medical Mycology (Kwon-Chung, KJ Bennett and JE Eds.), pp.464-513. Lea & Febiger, Philadelphia, 1992.
2) Chandler FW, Kaplan W, Ajello L: 13. Histoplasmosis capsulati, 14. Histoplasmosis duboisii, 15. Histoplasmosis farciminosi. *In* A Colour Atlas and Textbook of the Histopathology of Mycotic Diseases (Barry Carruthers, G. Eds.), pp.63-72. Wolfe Medical Publications, Ltd, Weert, Netherlands, 1980.
3) Tamura M, Kasuga T, Watanabe K, Katsu M, Mikami Y, Nishimura K: Phylogenetic characterization of *Histoplasma capsulatum* strains based on ITS region sequences, including two new strains from Thai and Chinese patients in Japan. Jpn J Med Mycol **43**: 11-19, 2002.
4) Kasuga T, Taylor JW, White TJ: Phylogenetic relationships of varieties and geographical groups of the human pathogenic fungus *Histoplasma capsulatum* Darling. J Clin Microbiol **37**: 653-663, 1999.
5) Kasuga T, White TJ, Koenig G, McEwen J,

Restrepo A, Castaneda E, da Silva Lacaz C, Heins-Vaccari EM, de Freitas RS, Zancope-Oliveira RM, Qin Z, Negroni R, Carter DA, Mikami Y, Tamura M, Taylor ML, Miller GF, Poonwan N, Taylor JW: Phylogeography of the fungal pathogen *Histoplasma capsulatum*. Mol Ecol **12**: 3383-3401, 2003.

6) Fell JW, Boekhout T, Fonseca A, Scorzetti G, Statzell-Tallman A: Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. Int J Syst Evol Microbiol **50**: 1351-1371, 2000.
7) Kurtzman CP, Robnett CJ: Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5' end of the large-subunit (26S) ribosomal DNA gene. J Clin Microb **35**: 1216-1223, 1997.
8) Peterson SW, Sigler L: Molecular genetic variation in *Emmonsia crescens* and *Emmonsia parva*, etiologic agents of adiaspiromycosis, and their phylogenetic relationship to *Blastomyces dermatitidis* (*Ajellomyces dermatitidis*) and other systemic fungal pathogens. J Clin Microbiol **36**: 2918-2925, 1998.
9) Leclerc MC, Philippe H, Guého E: Phylogeny of dermatophytes and dimorphic fungi based on large subunit ribosomal RNA sequence comparisons. J Med Vet Mycol **32**: 331-341, 1994.
10) Hall L, Wohlfiel S, Roberts GD: Experience with the MicroSeq D2 large-subunit ribosomal DNA sequencing kit for identification of commonly encountered, clinically important yeast species. J Clin Microbiol **41**: 5099-5102, 2003.
11) Sano A, Vilela MMS, Takahashi I, Fukushima K, Takizawa K, da Silva MTN, Uno J, Nishimura K, Miyaji M: Isolation of *Candida dubliniensis* from the oral cavity of an HIV-positive child in Brazil. Jpn J Med Mycol **41**: 177-181, 2000.
12) Higgins DG, Bleasby AJ, Fuchs R: CLUSTAL W: improved software for multiple sequence alignment. Comput Appl Biosci **8**: 189-191, 1992.
13) Sharmin S, Ohori A, Sano A, Kamei K, Yamaguchi M, Takeo K, Uno J, Nishimura K, Miyaji M: *Histoplasma capsulatum* variety *duboisii* isolated in Japan from an HIV-infected Ugandan patient. Jpn J Med Mycol **44**: 299-306, 2003.
14) Sugita T, Ikeda R, Shinoda T: Diversity among strains of *Cryptococcus neoformans* var. *gattii* as revealed by a sequence analysis of multiple genes and a chemotype analysis of capsular polysaccharide. Microbiol Immunol **45**: 757-768, 2001.
15) Kwon-Chung KJ: Sexual stage of *Histoplasma capsulatum*. Science **175**: 326, 1972.
16) Sugita T, Nishikawa A: Molecular taxonomy and identification of pathogenic fungi based on DNA sequence analysis. Jpn J Med Mycol **45**: 55-58, 2004.

Jpn. J. Med. Mycol.
Vol. 47, 113–117, 2006
ISSN 0916–4804

Short Report

Reexamination of *Coccidioides* spp. Reserved in the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Based on a Multiple Gene Analysis

Ayako Sano, Makoto Miyaji, Katsuhiko Kamei,
Yuzuru Mikami, Kazuko Nishimura

Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University,
1-8-1 Inohana, Chuo-ku, Chiba 260-8673, Japan

[Received: 4, November 2005. Accepted: 12, December 2005]

Abstract

The Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University is the only organization in Japan to possess a series isolates of *Coccidioides* spp., which are the most virulent pathogenic fungi and which are treated as biosafety level 3 microorganisms. Recently, the genus *Coccidioides* has been classified into two species, *C. immitis* and *C. posadasii*, based on their endemic areas and genotyping; the former species is endemic to the state of California, and the latter is endemic to other parts of North and South America. We reevaluated 19 isolates of *Coccidioides immitis* stored in our center using a multiple gene analysis. Five isolates were identified as *C. immitis* and 14 as *C. posadasii*. Their sequence information in GenBank will help to identify the two genospecies of *Coccidioides* spp.

Key words: *Coccidioides immitis*, *Coccidioides posadasii*, multiple gene analysis

Introduction

Coccidioides spp., the causative agents of coccidioidomycosis are the most virulent among pathogenic fungi. Coccidioidomycosis is treated as an imported fungal infection in Japan because almost all the patients were assumed to have gotten the infection in the United States or Mexico. It causes pneumonia and sometimes fatal systemic infection in healthy subjects, and is considered as a category-4 infection by the Ministry of Health, Labor, and Welfare of Japan. Clinicians who see patients with category-4 infections are required to report them to a local public health center[1].

The habitats of *Coccidioides* spp. are desert areas in North and South America. In Arizona, an endemic area, more than 2,000 new cases per year are reported and more than 100,000 individuals are infected with the fungus without symptoms[2]. In Japan, more than 45 cases had been recorded as of November 2005 (http://www.pf.chiba-u.ac.jp/). Most of the Japanese patients were infected in endemic areas to which they had traveled or resided, except for 1 case caused by contact with crude cotton imported from the United States. We could not determine the place of infection for some patients who had traveled to several endemic areas in The United States and Mexico.

The disease is caused by inhalation of arthroconidia of the fungi. The fungi grow as mycelia in spring and produce arthroconidia. They are easily spread into the air by the wind, by new construction activities, and by excavations. The clinical isolates of *Coccidioides* spp. on slants or plates are also easily diffused into the air and are apt to cause infections in laboratories. The culturing and identification of the fungi by mycological examination require at least 2 weeks for experts with special training[1].

C. immitis has been treated as a single species as the causative agent of coccidioidomycosis. Since 2002, *C. immitis* has been classified into

Address correspondence to: Ayako Sano
Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University
1-8-1 Inohana, Chuo-ku, Chiba 260-8673, Japan

two species, *C. immitis* and *C. posadasii*, based on the endemic areas and genotypes; the former species is endemic to the state of California, while the latter is endemic to other parts of North and South America[3]. In 1997, Koufopanou *et al.* tried to reclassify the species into two geographic types, a California type and an outside-California type, based on the combination of five gene analogues: chitin synthase, dioxygenase (DO), orotidine decarboxylase, serine proteinase (SP), and chitinase (CT)[4]. Fisher *et al.* created the two genospecies taxa based on the multiple gene analysis of microsatellites[3]. They also suggested a convenient method for the differentiation of *Coccidioides* spp. by a multiple gene analysis using a combination of three gene sequences: DO, SP, and CT[5]. The sequence data obtained from their investigations were not released to GenBank completely, because of patenting and bioterrorism crisis-management programs.

The present study aimed to reevaluate 19 strains formerly identified as *C. immitis* in our center following the criteria for genospecies proposed by Fisher *et al.* in 2002, to release their sequence data for public convenience, and to clarify the place of infection for Japanese patients. In addition to the genes used by Fisher *et al.*[3], we analyzed the internal transcribed spacer (ITS) 1-5.8S-ITS 2 and the D1/D2 regions of ribosomal RNA genes that are generally used as phylogenetic markers[6, 7] as well as a partial sequence of the urease gene (URE), which codes the virulence factor of pathogenic fungi[8]. The present study also tried to find specific genes to classify *Coccidioides* spp. from the genes named above.

Materials and Methods

Coccidioides spp. strains examined are shown in Table 1. They were formerly identified as *C. immitis* by the morphology observed through the test tubes, by the patient's history, or by identifications in the original country.

The fungi were cultured at 37°C for 2 weeks on potato dextrose agar (Difco, Franklin Lakes, NJ, USA) slants with a silicon plug. Then, a needle with a syringe containing 85% ethanol was pierced the plug and ethanol was slowly injected until the plug was immersed. The point of needle insertion into the plug was covered with a mass of cotton soaked with 70% ethanol. The fungal mass was fixed by a final concentration of approximately 70% ethanol for 48 hours at room temperature. A loop of fungal mass was spread on a PDA plate and cultured at 37°C for 7 days to check its survival. The next step was started after we confirmed that the fungus had no sprouts. The above procedures were done in a special laboratory for level 3 biohazard pathogens.

DNA was extracted with a DEXPAT® Kit (TaKaRa, Ohtsu, Japan) with a modification of the manufacturer's protocol. Approximately 100 μl of the fungal mass was transferred to a sterilized microtube (1.5 ml), homogenized with 0.5 ml of DEXPAT® solution by a plastic pestle. The mixture was incubated at 100°C for 10 min and centrifuged at 12,000 rpm (13,201 g) for another 10 min. The supernatant was used

Table 1. *Coccidioides* spp. isolates and their accession numbers

Isolate					Gene (length)				
IFM No.	Strain	Identification	Origin	Country	Deoxygenase (636)	Serine proteinase (646)	Chitinase (487)	Urease (536)	rRNA (1251)
4935	Nagoya	*C. posadasii*	Human case	Japan	AB232864	AB232726	AB232745	AB232707	AB232883
4945	Ohashi	*C. posadasii*	Human case	Japan	AB232865	AB232727	AB232746	AB232708	AB232884
45809	Silviera (SAP2)	*C. posadasii*	Animal passage 11	USA	AB232866	AB232728	AB232747	AB232709	AB232885
45810	Silveira (SAP3)	*C. posadasii*	Animal passage 111	USA	AB232867	AB232729	AB232748	AB232710	AB232886
45811	Arizona	*C. posadasii*	Human case	USA	AB232868	AB232730	AB232749	AB232711	AB232887
45812	San Antonio	*C. posadasii*	Human case	USA	AB232869	AB232731	AB232750	AB232712	AB232888
45813	New York	*C. posadasii*	Human case	USA	AB232870	AB232732	AB232751	AB232713	AB232889
45815	91-48	*C. immitis*	Human case	USA	AB232871	AB232733	AB232752	AB232714	AB232890
45816	91-153	*C. immitis*	Human case	USA	AB232872	AB232734	AB232753	AB232715	AB232891
45817	Nicols	*C. posadasii*	Human case	USA	AB232873	AB232735	AB232754	AB232716	AB232892
46868	Yokohama	*C. immitis*	Human case	Japan	AB232874	AB232736	AB232755	AB232717	AB232893
50992	90-242	*C. immitis*	Human case	USA	AB232875	AB232737	AB232756	AB232718	AB232894
50993	Kanazawa	*C. posadasii*	Human case	Japan	AB232876	AB232738	AB232757	AB232719	AB232895
50994	Toranomon	*C. posadasii*	Human case	Japan	AB232877	AB232739	AB232758	AB232720	AB232896
50995	Handai	*C. immitis*	Human case	Japan	AB232878	AB232740	AB232759	AB232721	AB232897
51112	Himeji	*C. posadasii*	Human case	Japan	AB232879	AB232741	AB232760	AB232722	AB232898
54194	Nagano	*C. posadasii*	Human case	Japan	AB232880	AB232742	AB232761	AB232723	AB232899
54195	Fukunaga	*C. posadasii*	Human case	Japan	AB232881	AB232743	AB232762	AB232724	AB232900
54196	Chiba	*C. posadasii*	Human case	Japan	AB232882	AB232744	AB232763	AB232725	AB232901

as the DNA sample[9]. DNA extract (2.5 μl), Ready-to-Go beads (Amersham Pharmacia, Tokyo, Japan), 20 μl of distilled water, and 2.5 μl of 10 pM of each primer for DO, SP, CT, and rRNA genes[4-7] were mixed, and polymerase chain reactions (PCR) were processed. PCR products were separated by electrophoresis on 1.0% agarose gels in 1 × TBE buffer (0.04 M Tris-boric acid, 0.001 M EDTA pH 8.0) and visualized by ethidium bromide staining. PCR products were purified with a PCR purification kit (QIAquick®, Qiagen) and labeled using BigDye® terminator Ver. 1.1 (Applied Biosystems, Foster City, CA, USA) by the manufacturer's protocol.

The partial sequence of the urease gene (URE) was amplified with a primer set (CIS2410-2434: CGG GTA TTT ACA AGG CTG ATA TTG G and CIAS2945-2922: GAA GCC AGA TTC GTT CAG GGT GTC) designed from the *C. immitis* urease gene sequence deposited in GenBank under accession number U81509[8]. The PCR conditions for URE were as follows: the reaction mixture was subjected to 1 cycle of denaturation at 95°C for 4 min, 30 cycles of amplification at 94°C for 1 min, at 50°C for 1 min, and at 72°C for 2 min, and a final extension cycle at 72°C for 10 min with a PCR Thermal Cycler MP (TaKaRa).

The combined data set for phylogenic study was performed in *Fusarium* spp.[10]. The present study applied above analysis for alignment of combined sequences consisted of at least 1,769 base pairs obtained from the DO, SP, and CT genes using CLUSTAL X (Version 1.8)[11]. An unrooted tree was constructed using Njplot (http://pbil.univ-lyon1.fr/software/njplot.html)[12]. The trees were also constructed based on each gene alone. Consistencies in clade formation between the unrooted trees based on each gene and the combined one were compared.

The genospecies of *Coccidioides* were determined based on the location of the clade. Strains located in the clade involving IFM 50995 derived from a Japanese patient whose infection was suspected of having occurred in Bakersfield, California[13] was identified as *Coccidioides immitis*, and those in the clade with IFM 45811 and IFM 45812 originating in the state of Arizona were identified as *Coccidioides posadasii*, respectively.

Results and Discussion

The accession numbers of the genes, the lengths of the sequences, and the identification based on the cluster analysis with the three-gene combination are shown in Table 1. The unrooted tree based on a combination of three genes is shown in Fig. 1. Five isolates of *Coccidioides* spp. in our center were identified as

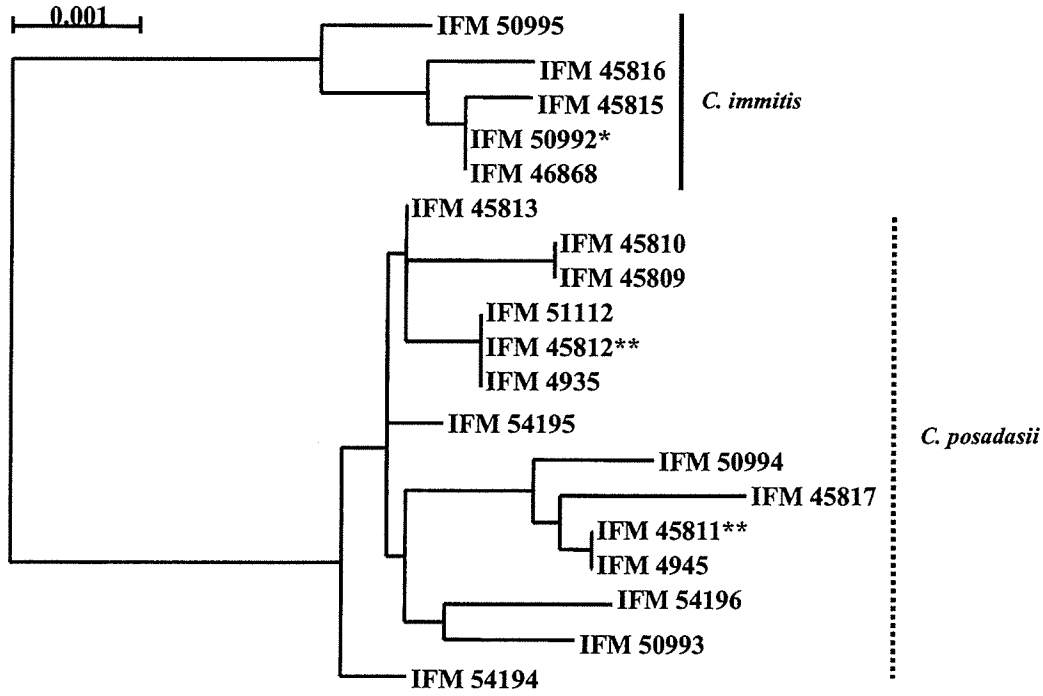

Fig. 1. Unrooted tree based on a combination of three gene sequences–dioxygenase (DO), serine proteinase (SP), and chitinase (CT) consisting of 1769 base pairs constructed by the neighbor-joining method. The scale bar represents a difference corresponding to 0.001 (0.1%). Isolates identified as *C. immitis* are in the upper clade and those as *C. posadasii* are in the lower one. *: isolate used as *C. immitis* standard; **: isolate used as *C. posadasii* standard.

C. immitis and 14 as *C. posadasii*. The data confirmed that 2 of the Japanese cases were caused by *C. immitis*.

Unrooted trees based on individual DO, SP, and URE genes were consistent with the combination of three genes (DO, SP, and CT) and could separate two clades, while those of CT and rRNA genes could not (data not shown). The homology among isolates was more than 99% in all genes evaluated.

The GenBank database was not sufficient for genetic identification of *Coccidioides* spp. There was one sequence of DO on *C. posadasii* (L38493), four sequences of SP on *C. immitis* (S77562, AJ408857, AJ408861, and M81863) and four sequences on *C. posadasii* (AJ408858, AJ408859, AJ408860, and X63114), three sequences of CT on *C. posadasii* (L41663, U51271, and U60806), one sequence of URE on *C. posadasii* (U81509), two sequences of the ITS region of rRNA on *C. posadasii* (CIU18360, X94142), and two sequences of the D1/D2 region of rRNA on *C. immitis* (AY176713, AB040702). The gene sequences of the species used for the present study are helpful for genetic identification. Cluster analysis in the present study with a combination of these data found an inconsistency on the SP gene. The sequence AJ408857, derived from *C. immitis* in the GenBank database, was located on the cluster of *C. posadasii*.

Thus far, two genes, DO and URE, might be useful for identifying genospecies of *Coccidioides* spp. alone. Such an analysis will allow speculation on where infection occured, while both the ITS and D1/D2 regions of rRNA genes, which are representative genetic markers for classifying and identifying fungal species[6, 7], were incompatible with the unrooted tree based on the cluster analysis by combination of the three genes. Identification based on ribosomal RNA genes could not identify the species of *Coccidioides* because of strongly similar identity among strains according to the criteria proposed by Kurtzman and Robnett[7].

The inter species differences between *C. immitis* and *C. posadasii* linked both geographic distribution and virulence[5]. However, clinical isolates of both *C. immitis* and *C. posadasii* in Japan should be regarded as the most virulent fungal species. We should keep in mind that the clinical isolates of *Coccidioides* spp. should be handled in accordance with biohazard regulations at a bio-safety level 3 laboratory.

Acknowledgements

The present study was supported by the National Bioresource Project supported by the Ministry of Education, Culture, Sports, Science, and Technology, Japan, and by the Special Research Fund for Emerging and Re-emerging Infections by the Ministry of Health, Labor, and Welfare, Japan.

References

1) Kamei K: Imported mycoses in Japan: Their present status and problems. Jpn J Med Mycol **46**: 17-20, 2005.
2) Cox RA, Magee DM: Coccidioidomycosis: host response and vaccine development. Clin Microbiol Rev **17**: 804-839, 2004.
3) Fisher MC, Koenig GL, White TJ, Taylor JW: Molecular and phenotypic description of *Coccidioides posadasii* sp. nov., previously recognized as the non-California population of *Coccidioides immitis*. Mycologia **94**: 73-84, 2002.
4) Koufopanou V, Burt A, Taylor JW: Concordance of gene genealogies reveals reproductive isolation in the pathogenic fungus *Coccidioides immitis*. Proc Natl Acad Sci USA **94**: 5478-5482, 1997.
5) Fisher MC, Koenig GL, White TJ, San-Blas G, Negroni R, Alvarez IG, Wanke B, Taylor JW: Biogeographic range expansion into South America by *Coccidioides immitis* mirrors new world patterns of human migration. Proc Natl Acad Sci USA **98**: 4558-4562, 2001.
6) White TJ, Bruns T, Lee S, Taylor J: Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In*: M.A. Innis, D.H. Gelfard, J.J. Sniinski, and T.J. White, Editors, PCR protocols: A guide to methods and applications, Academic Press Inc., San Diego, CA, pp.315-322, 1990.
7) Kurtzman CP, Robnett CJ: Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5' end of the large-subunit (26S) ribosomal DNA gene. J Clin Microb **35**: 1216-1223, 1997.
8) Yu JJ, Smithson SL, Thomas PW, Kirkland TN, Cole GT: Isolation and characterization of the urease gene (URE) from the pathogenic fungus *Coccidioides immitis*. Gene **198**: 387-391, 1997
9) Endo S, Komori T, Ricci G, Sano A, Yokoyama K, Ohori A, Kamei K, Franco M, Miyaji M, Nishimura K: Detection of gp43 of *Paracoccidioides brasiliensis* by the loop-mediated isothermal amplification (LAMP) method. FEMS Microbiol Lett. **234**: 93-97, 2004.
10) Aoki T, O'Donnell K, Scandiani MM: Sudden death syndrome of soybean in South America is caused by four species of *Fusarium*: *Fusarium brasiliense* sp. nov., *F. cuneirostrum* sp. nov., *F.*

tucumaniae, and *F. virguliforme*. Mycosciense **46**: 162-183, 2005.
11) Jeanmougin F, Thompson JD: Multiple sequence alignment with Clustal X. Trends Biochem Sci **23**: 403-405, 1998.
12) Perrière G, Gouy M: WWW-Query: an on-line retrieval system for biological sequence banks. Biochimie **78**: 364-369, 1996.
13) Osaki T, Morishita H, Maeda H, Kamei K, Hoshino S, Kijima T, Kumagai T, Yoshida M, Tachibana I, Kawase I: Pulmonary coccidioidomycosis that formed a fungus ball with 8-years duration. Internal Medicine **44**: 141-144, 2005.

JOURNAL OF CLINICAL MICROBIOLOGY, May 2006, p. 1859–1862 Vol. 44, No. 5
0095-1137/06/$08.00+0 doi:10.1128/JCM.44.5.1859–1862.2006

Novel Approach to Designing Primers for Identification and Distinction of the Human Pathogenic Fungi *Coccidioides immitis* and *Coccidioides posadasii* by PCR Amplification

Takashi Umeyama,[1] Ayako Sano,[2] Katsuhiko Kamei,[2] Masakazu Niimi,[1] Kazuko Nishimura,[2] and Yoshimasa Uehara[1]*

Department of Bioactive Molecules, National Institute of Infectious Diseases, Tokyo 162-8640,[1] and Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Chiba 260-8673,[2] Japan

Received 10 January 2006/Returned for modification 28 February 2006/Accepted 3 March 2006

We developed a pair of primers that specifically identifies *Coccidioides* species, etiologic agents of the human fungal disease coccidioidomycosis. These primers could be used for distinguishing *Coccidioides immitis* and *Coccidioides posadasii* by simply comparing the amplicon sizes on an agarose gel.

Coccidioidomycosis, a fungal respiratory disease of humans caused by *Coccidioides* species, is endemic to arid areas of the Americas. Two species of *Coccidioides* are now recognized, whereas until recently, coccidioidomycosis was attributed to only one species, *Coccidioides immitis* (4). *Coccidioides posadasii*, formerly known as the non-California *C. immitis* strain, is found mainly in Texas, Arizona, and regions of endemicity outside of the United States, whereas *C. immitis* is found primarily in the Central Valley of California. These two species can be divided based on single-nucleotide polymorphisms and the size of microsatellites (3, 4), although the colony morphologies, growth rates, and clinical presentations are almost identical. Because identification of *Coccidioides* spp. carries with it a great deal of risk, molecular diagnosis without culturing has long been expected. Many researchers have explored nucleic acid detection for the diagnosis of coccidioidomycosis (2, 7–9, 11, 12).

For isolates of *C. immitis* and *C. posadasii*, listed in Table 1, the same DNA samples previously described by Sano et al. (10) were used. For non-*Coccidioides* fungal isolates, listed in Table 2, DNA isolation was performed using a DEXPAT DNA extraction kit (Takara Bio Inc., Japan). PCR was performed with approximately 10 ng of extracted DNA in a 20-μl reaction volume consisting of LA *Taq* buffer II (Mg^{2+} plus) (Takara Bio Inc.), 200 μM deoxynucleoside triphosphates, 2.5 U of ExTaq DNA polymerase (Takara Bio Inc.), and 10 pmol of each primer. One cycle at 94°C for 3 min followed by 35 cycles at 94°C for 30 s, at 60°C for 30 s, and at 72°C for 45 s with a final extension step at 72°C for 3 min was performed in a PTC-200 DNA Engine thermal cycler (Bio-Rad). The amplified DNA must be handled carefully in order to avoid amplicon contamination. The universal fungal primers ITS1 and ITS4 were used in all DNA samples to verify the efficiency of the test and to ensure that there was no PCR inhibition in the DNA samples (8). Ten microliters of each PCR product was electrophoresed through 2% agarose gel and visualized with a UV light after ethidium bromide or SYBR Safe (Invitrogen) staining. The PCR products were purified from gel with a NucleoSpin Extract II kit (Macherey-Nagel, Germany). Nucleotide sequences were determined using a BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems) and an ABI 3130 genetic analyzer (Applied Biosystems).

Empirically, the construction of diagnostic primers is based on a nucleotide sequence encoding conserved enzymes or rRNA. However, we left the matter to chance: we repeated the primer construction based on randomly selected regions and verification by actual PCR experiments. In step 1, we obtained a text file containing the *C. immitis* genome sequence (http://www.broad.mit.edu/annotation/fungi/coccidioides_immitis/). A nucleotide sequence corresponding to the 240- to 720-bp length was randomly selected from the genome database file. We designed 20-mer forward and reverse primers, which were expected to amplify the randomly selected region. In step 2, we examined whether these two primers could amplify DNA fragments of the anticipated size from *Coccidioides* spp. In step 3, we tested whether the primers that could amplify *Coccidioides* DNA in step 2 were unable to amplify *Candida albicans* and *Aspergillus fumigatus* DNA. By repeating these steps on 64 selected regions, we nominated one pair of primers for more detailed examination. Since this experimental primer design led to our successful product, mentioned below, this strategy should be a powerful method for the development of diagnostic primers.

The selected primers were Coi9-1F (5′-TACGGTGTAATC CCGATACA-3′) and Coi9-1R (5′-GGTCTGAATGATCTG ACGCA-3′). The selected primer set was constructed to amplify a 720-bp amplicon that corresponds to nucleotide position 660313 to 661032 of *C. immitis* contig 2.2 (accession number AAEC02000002). Nineteen isolates of *Coccidioides* spp. were examined for the developed primers (Table 1). The PCR system with the specific primer pairs was able to amplify the DNA fragment of the expected size from DNAs of *C. immitis* (Fig. 1). For specificity testing, 137 isolates of 52 fungal species were examined (Table 2). As a result of PCR using DNAs from these fungi, the primers were proved not to cross-amplify with major pathogenic fungi and related ones, such as *Arthrographis kalrae*, *Chrysosporium* spp., *Geotrichum candidum*, *Malbranchea* spp.,

* Corresponding author. Mailing address: Department of Bioactive Molecules, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. Phone: 81 3 5285-1111. Fax: 81 3 5285-1175. E-mail: yuehara@nih.go.jp.

TABLE 1. *Coccidioides* isolates used in this study

Lane no. in Fig. 1	Species	IFM no.[a]	Origin
1	*C. posadasii*	4935	Japan
2	*C. posadasii*	4945	Japan
3	*C. posadasii*	45809	United States
4	*C. posadasii*	45810	United States
5	*C. posadasii*	45811	United States
6	*C. posadasii*	45812	United States
7	*C. posadasii*	45813	United States
8	*C. immitis*	45815	United States
9	*C. immitis*	45816	United States
10	*C. posadasii*	45817	United States
11	*C. immitis*	46868	Japan
12	*C. immitis*	50992	United States
13	*C. posadasii*	50993	Japan
14	*C. posadasii*	50994	Japan
15	*C. immitis*	50995	Japan
16	*C. posadasii*	51112	Japan
17	*C. posadasii*	54194	Japan
18	*C. posadasii*	54195	Japan
19	*C. posadasii*	54196	Japan

[a] IFM, Institute for Food Microbiology, Chiba University, Chiba, Japan.

TABLE 2. Fungal species used as negative controls in this study

Species	No. of tested isolates
Absidia sp.	1
Alternaria sp.	1
Apinisia spp.	2
Arthroderma spp.	8
Arthrographis spp.	4
Aspergillus spp.	5
Auxarthron sp.	1
Basidiobolus sp.	1
Blastomyces sp.	1
Candida spp.	8
Chrysosporium spp.	4
Cladophialophora spp.	2
Cokeromyces sp.	1
Conidiobolus sp.	1
Cryptococcus spp.	5
Cunninghamella sp.	1
Emmonsia spp.	3
Epidermophyton sp.	1
Exophiala spp.	4
Fonsecaea sp.	1
Fusarium spp.	2
Geotrichum sp.	1
Gymnoascoideus spp.	3
Gymnoascus spp.	5
Histoplasma spp.	6
Hortaea sp.	1
Malassezia spp.	2
Malbranchea spp.	17
Microsporum spp.	2
Mortierella sp.	1
Mucor spp.	2
Neosartorya sp.	1
Paecilomyces spp.	2
Paracoccidioides spp.	6
Penicillium spp.	7
Phanerochaete spp.	2
Phialophora spp.	2
Prototheca sp.	1
Pseudallescheria sp.	1
Rhinocladiella sp.	1
Rhizomucor sp.	1
Rhizopus spp.	3
Scedosporium sp.	1
Schizophyllum sp.	2
Scopulariopsis sp.	1
Sporothrix sp.	1
Syncephalastrum sp.	1
Trichophyton spp.	3
Trichosporon sp.	1
Uncinocarpus sp.	1
Veronaea sp.	1
Zygorhynchus sp.	1

Paracoccidioides brasiliensis, and *Trichosporon asahii*. The *Coccidioides* diagnostics based on a proline-rich antigen (2, 6) or internal transcribed spacer region (5, 7) have been reported so far. However, Bialek (1) has pointed out that the possibility of cross-amplification of human and murine DNA by the ITS primers (7) was not excluded. Since no amplification of human DNA by the primers in this report was detected (data not shown), we have developed a useful PCR system applicable for use in clinical diagnosis.

Surprisingly, two different mobilities of the DNA fragment were observed (Fig. 1) when the primers were tested for all *Coccidioides* spp. DNA available in Japan (Table 1). The DNA fragment amplified from *C. posadasii* was obviously shorter than that from *C. immitis*. Nucleotide sequence analysis of the amplified DNA revealed that the amplicon from *C. posadasii* had a contiguous deletion of 86 bp compared to that from *C. immitis* (Fig. 2). Therefore, such a large deletion contributed to the convenient distinction of two very close species, *C. immitis* and *C. posadasii*, which had previously been difficult to distinguish. In actual distinction on an agarose gel, standard amplicons of *C. immitis* and *C. posadasii* should be run as controls

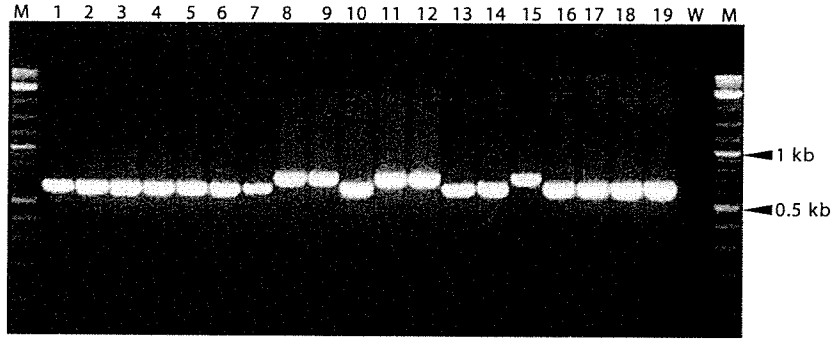

FIG. 1. PCR amplification of coccidioidal DNAs. Lanes M, DNA molecular weight marker used to estimate product size; lane W, distilled water used as a negative control; lanes 1 to 7, 10, 13, 14, and 16 to 19, *C. posadasii*; lanes 8, 9, 11, 12, and 15, *C. immitis*. The exact description of *Coccidioides* spp. is in Table 1.

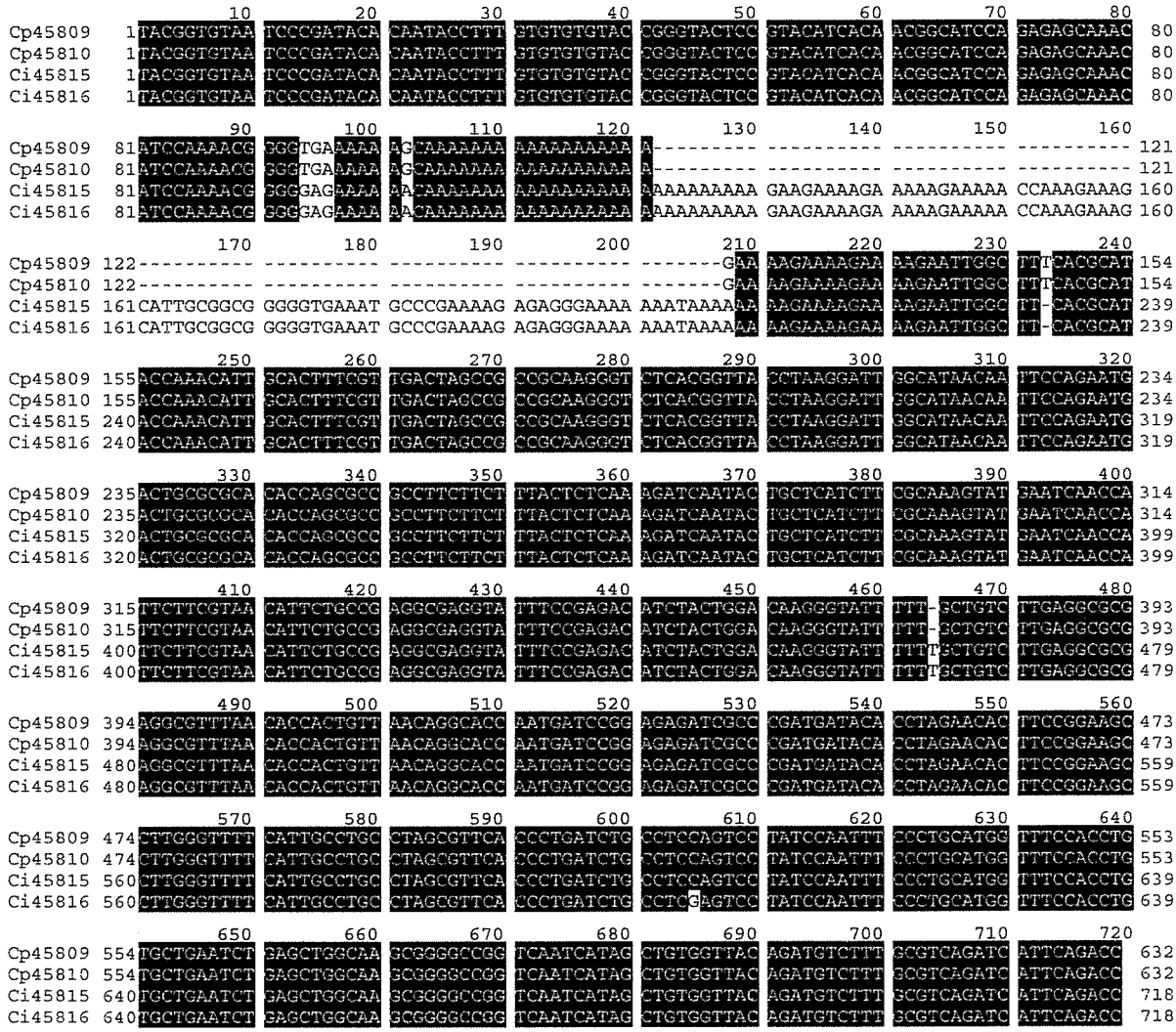

FIG. 2. Nucleotide sequence alignment of DNA fragments amplified with Coi9-1F and Coi9-1R primers between *C. posadasii* IFM45809 (Cp45809) and IFM45810 (Cp45810) and *C. immitis* IFM45815 (Ci45815) and IFM45816 (Ci45816).

because of the close proximity of the two bands. Two methods for differentiating *C. immitis* and *C. posadasii* are currently being used: the lengths of the microsatellite loci and the single-nucleotide polymorphisms within several enzymes (3). Both methods require highly skilled molecular biological techniques at a relatively high cost. The PCR system with the primers we developed might facilitate operation and provide high-throughput handling. Thus, this will provide high value in epidemiology, such as tracing the route of laboratory-acquired infection or analyzing a pandemic that might occur in the future. Whether these primers can be clinically applied remains to be seen. Further development will contribute to the early diagnosis of coccidioidomycosis.

We gratefully acknowledge the excellent technical assistance of Yuki Utena-Abe.

This study was supported by Health and Labour Sciences Research Grants for Research on Emerging and Re-emerging Infectious Diseases from Japan's Ministry of Health, Labour and Welfare. This study was also supported by the National Bioresource Project-Pathogenic Microorganisms of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

1. **Bialek, R.** 2005. Amplification of coccidioidal DNA in clinical specimens by PCR. J. Clin. Microbiol. **43**:1492–1493.
2. **Bialek, R., J. Kern, T. Herrmann, R. Tijerina, L. Cecenas, U. Reischl, and G. M. Gonzalez.** 2004. PCR assays for identification of *Coccidioides posadasii* based on the nucleotide sequence of the antigen 2/proline-rich antigen. J. Clin. Microbiol. **42**:778–783.
3. **Fisher, M. C., G. L. Koenig, T. J. White, G. San-Blas, R. Negroni, I. G. Alvarez, B. Wanke, and J. W. Taylor.** 2001. Biogeographic range expansion into South America by *Coccidioides immitis* mirrors New World patterns of human migration. Proc. Natl. Acad. Sci. USA **98**:4558–4562.
4. **Fisher, M. C., G. L. Koenig, T. J. White, and J. W. Taylor.** 2002. Molecular and phenotypic description of *Coccidioides posadasii* sp. nov., previously recognized as the non-California population of *Coccidioides immitis*. Mycologia **94**:73–84.
5. **Greene, D. R., G. L. Koenig, M. C. Fisher, and J. W. Taylor.** 2000. Soil isolation and molecular identification of *Coccidioides immitis*. Mycologia **92**:406–410.
6. **Johannesson, H., P. Vidal, J. Guarro, R. A. Herr, G. T. Cole, and J. W. Taylor.** 2004. Positive directional selection in the proline-rich antigen (PRA) gene among the human pathogenic fungi *Coccidioides immitis*, *C. posadasii* and their closest relatives. Mol. Biol. Evol. **21**:1134–1145.

7. **Johnson, S. M., K. A. Simmons, and D. Pappagianis.** 2004. Amplification of coccidioidal DNA in clinical specimens by PCR. J. Clin. Microbiol. **42:**1982–1985.
8. **Lindsley, M. D., S. F. Hurst, N. J. Iqbal, and C. J. Morrison.** 2001. Rapid identification of dimorphic and yeast-like fungal pathogens using specific DNA probes. J. Clin. Microbiol. **39:**3505–3511.
9. **Sandhu, G. S., B. C. Kline, L. Stockman, and G. D. Roberts.** 1995. Molecular probes for diagnosis of fungal infections. J. Clin. Microbiol. **33:**2913–2919.
10. **Sano, A., M. Miyaji, K. Kamei, Y. Mikami, and K. Nishimura.** Reexamination of *Coccidioides* spp. reserved in the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, based on a multiple gene analysis. Nippon Ishinkin Gakkai Zasshi (Jpn. J. Med. Mycol.), in press.
11. **Shubitz, L., T. Peng, R. Perrill, J. Simons, K. Orsborn, and J. N. Galgiani.** 2002. Protection of mice against *Coccidioides immitis* intranasal infection by vaccination with recombinant antigen 2/PRA. Infect. Immun. **70:**3287–3289.
12. **Stockman, L., K. A. Clark, J. M. Hunt, and G. D. Roberts.** 1993. Evaluation of commercially available acridinium ester-labeled chemiluminescent DNA probes for culture identification of *Blastomyces dermatitidis*, *Coccidioides immitis*, *Cryptococcus neoformans*, and *Histoplasma capsulatum*. J. Clin. Microbiol. **31:**845–850.

Jpn. J. Med. Mycol.
Vol. 47, 225−229, 2006
ISSN 0916−4804

Original Article

A One-Enzyme PCR-RFLP Assay for Identification of Six Medically Important *Candida* Species

Hossein Mirhendi[1], Koichi Makimura[2], Mohamadreza Khoramizadeh[3], Hideyo Yamaguchi[2]

[1]Department of Medical Parasitology and Mycology, School of Public Health and Institute of Public Health Researches (Esfahan Center), Tehran University of Medical Sciences, Tehran, IRAN
[2]Institute of Medical Mycology and Genome Research Center, Teikyo University, 359 Otsuka, Hachioji, Tokyo 192-0395, Japan
[3]Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, IRAN

[Received: 17, November 2005. Accepted: 17, April 2006]

Abstract

Early identification of *Candida* isolates to the species level is necessary for effective antifungal therapy, and can also facilitate control of hospital infections. Phenotype-based methods for identifying *Candida* species are often difficult and time-consuming. Molecular biological techniques provide a useful alternative approach. In the present study, the ITS1-5.8S-ITS2 regions of fungal rRNA genes were amplified with universal primers in 20 standard strains. Digestion of the PCR products with one restriction enzyme, *Msp*I, allowed discrimination of medically important *Candida* species, including *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*, and *C. guilliermondii*. Using this method, we successfully identified 137 clinical isolates of *Candida*. Among them, *C. albicans* was identified as the most common species, followed by *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, *C. krusei*, and *C. guilliermondii*. This method is a simple, rapid, and cost-effective method for differentiation between species that is applicable in clinical laboratories.

Key words: *Candida*, PCR, RFLP, *Msp*I, identification

Introduction

Candida species are considered a major cause of opportunistic infections in humans. In recent years, despite advances in health care and therapeutic methods, the incidence of invasive systemic candidiasis has increased markedly. This is thought to be the result of the increase in size of populations at risk, such as transplant recipients, cancer patients, HIV-infected patients, and those receiving immunosuppressive and broad-spectrum antibiotic therapy[1-3]. Although the majority of *Candida* infections are caused by *C. albicans*, non-*albicans* species of *Candida*, such as *C. glabrata* and *C. krusei*, which are less susceptible to azoles derivatives have been reported with increasing frequency[4, 5]. Early diagnosis of invasive fungal infections is essential to reduce the mortality rates. In addition, identification of species is essential for effective antifungal therapy with regard to the emergence of resistance to antifungal drugs. Although *Candida* identification kits based on assimilation tests are commercially available, these kits require at least 1 to 5 days for identification of *Candida* at the species level. Recently, molecular techniques have provided alternative methods for diagnosis and identification of pathogenic fungi, including *Candida* species[6, 7]. Molecular methods with high discriminatory power are required for reliable identification of *Candida* at the species level, especially in epidemiological studies to assess the transmission routes as well as to determine appropriate antifungal drugs[8-10]. Although various methods have been reported for molecular identification of *Candida* spp.,

Corresponding author: Hossein Mirhendi, PhD.
epartment of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, 14155-6446, IRAN

such as RAPD (random amplified polymorphic DNA)-PCR, DNA sequence analysis, and mitochondrial large subunit ribosomal RNA gene sequencing[11, 12], these tests are still time-consuming and too expensive for routine use, especially in medical laboratories. Here, we report the application of a rapid PCR-based technique using a one-enzyme restriction fragment length polymorphism (RFLP), for discrimination of six clinically important *Candida* species.

Materials and Methods

Strains: Standard strains, which were provided by Teikyo University Institute of Medical Mycology (TIMM), Tokyo, Japan, are listed in Table 1. In addition, 137 clinical isolates from the Medical Mycology Laboratory, Tehran University of Medical Sciences, Iran, were also used. All yeasts were cultured on Sabauroud's dextrose agar and incubated at 32°C for two days. All clinical isolates were also cultured on CHROMagar *Candida* (Kanto Chemical Co., Ltd., Tokyo) and incubated at 35°C for 48 h for production of species-specific colors.

DNA extraction: Genomic DNA was extracted and purified using glass bead disruption[13]. Briefly, a loop full of fresh yeast was harvested and suspended in 300 μl of lysis buffer (10 mM Tris, 1 mM EDTA pH 8, 1% SDS, 100 mM NaCl, 2% Triton X-100). After adding 300 μl of phenol-chloroform (1 : 1) and 300 mg of glass beads (0.5 mm in diameter), samples were vortexed vigorously for 5 min to disrupt the cells completely. Cellular debris was separated by centrifugation at 10,000 rpm for 5 min and the aqueous layer was extracted once more with an equal volume of chloroform. Total DNA in the supernatant was precipitated with 2-propanol, washed with 70% ethanol, air-dried, resuspended in 100 μl of TE buffer (10 mM Tris, 1 mM EDTA), and preserved at −20°C until use.

PCR conditions: PCR amplification was carried out in a final volume of 100 μl. Each reaction contained 1 μl of template DNA, each forward (ITS1, 5'-TCC GTA GGT GAA CCT GCG G-3') and reverse (ITS4, 5'-TCC TCC GCT TAT TGA TAT GC-3') primer at 0.2 μM, each deoxynucleoside triphosphate (dNTP) at 0.1 mM, 10 μl of 10× PCR buffer, and 2.5 U of *Taq* DNA polymerase. An initial denaturation step at 94°C for 5 min was followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 45 s, and extension at 72°C for 1 min, with a final extension step of 72°C for 7 min. Amplified products were visualized by 1.5% (w/v) agarose gel electrophoresis in TBE buffer (0.09 M Tris, 0.09 M boric acid, and 20 mM EDTA, pH 8.3),

Table 1. Standard yeast strains used in the present study

Species	Strains
Candida albicans	ATCC 10261, ATCC 10231, ATCC 24432, TIMM 1768
C. glabrata	ATCC 90030, CBS 138
C. tropicalis	ATCC 0750, TIMM 0313
C. krusei	ATCC 6258, TIMM 3404
C. parapsilosis	ATCC 22019, ATCC 90018
C. guilliermondii	ATCC 9058, TIMM 0257
C. albicans var. *stellatoidea*	TIMM 1309
C. dubliniensis	CBS 7987
Cryptococcus neoformans	ATCC 90113
Saccharomyces cerevisiae	ATCC 9763, ATCC 2366
Trichosporon asahii	TIMM 3411

Table 2. Sizes of ITS1-ITS4 PCR products for *Candida* species before and after digestion with *Msp* I

Candida species	Size of ITS1-ITS4	Size (s) of restriction product (s)	*Accession number
C. albicans	535	297, 238	L47111
C. glabrata	871	557, 314	AF167993
C. tropicalis	524	340, 184	L47112
C. krusei	510	261, 249	L47113
C. guilliermondii	608	371, 155, 82	L47110
C. parapsilosis	520	520	L47109

*DDBG/EMBL/GenBank accession number

stained with ethidium bromide (0.5 μg ml^{-1}), and photographed.

RFLP analysis: The ITS1-ITS4 sequences of various *Candida* species obtained from DDBJ/EMBL/GenBank databases were aligned and restriction patterns of the PCR products of the species mentioned above were predicted for each of the known restriction enzymes using DNASIS software (Hitachi Software Engineering Co., Tokyo). Predicted restriction fragments were compared to choose the best discrimination. Finally, the enzyme *Msp*I was selected to achieve the best species-specific length patterns as shown in Table 2. Digestion was performed by incubating a 20-μl aliquot of PCR product with 10 U of *Msp*I (Roche Molecular, Mannhiem, Germany) in a final reaction volume of 25 μl at 37°C for 2 h. Restriction fragments were separated by 1.8% agarose gel electrophoresis in TBE buffer for approximately 45 min at 100 V and visualized by staining with ethidium bromide.

Results

Fungus-specific universal primer pairs (ITS1 and ITS4) were able to successfully amplify the ITS region of all yeasts tested, providing a single PCR product of approximately 510~870 bp (Fig. 1). After analysis of various restriction enzymes, *Msp*I was selected as the best enzyme

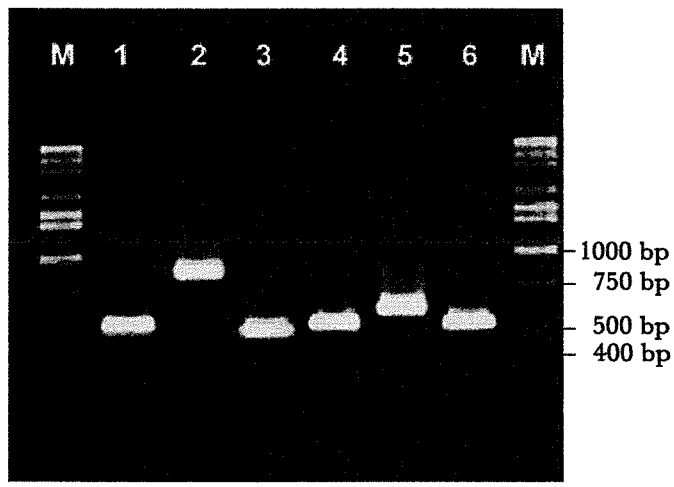

Fig. 1. PCR products from six *Candida* species. Lanes 1-6: *C. albicans* (ATCC 10261), *C. glabrata* (ATCC 90030), *C. tropicalis* (ATCC 0750), *C. krusei* (ATCC 6258), *C. guilliermondii* (ATCC 9058) and *C. parapsilosis* (ATCC 22019), respectively. Lanes M: Molecular size marker

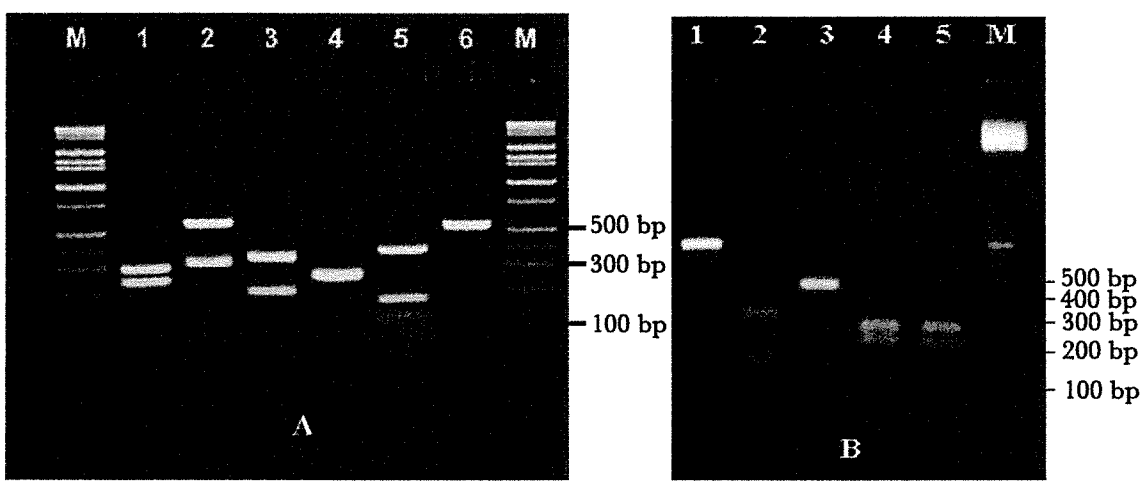

Fig. 2. A) Restriction digestion of PCR products of *Candida* strains with the enzyme *Msp*I. Lanes 1-6: *C. albicans* (ATCC 10261), *C. glabrata* (ATCC 90030), *C. tropicalis* (ATCC 0750), *C. krusei* (ATCC 6258), *C. guilliermondii* (ATCC 9058) and *C. parapsilosis* (ATCC 22019), respectively. Lane M: molecular size marker. B) Restriction digestion of PCR products of other strains with the enzyme *Msp*I. Lanes 1-5: *S. cerevisiae* (ATCC 9763), *T. asahii* (TIMM 3411), *C. neoformans* (ATCC 90113), *C. albicans* var. *stellatoidea* (TIMM1309) and *C. dubliniensis* (CBS 7987), respectively. Lane M: Molecular size marker

Table 3. Identification of clinical isolates of *Candida* species in Iran by PCR-RFLP amplification of the ITS1-ITS4 region and RFLP analysis with *Msp*I

Species	Number	%
C.albicans and/or *C. dubliniensis*	93	67.9
C. tropicalis	12	8.8
C. parapsilosis	12	8.8
C. glabrata	9	6.6
C. krusei	7	5.1
C. guilliermondii	4	2.9
Total	137	100

for differentiation between six medically important *Candida* species. PCR amplicons were digested with *Msp*I as described in Materials and Methods. The products of digestion are shown separately in Fig. 2, which shows that the bands generated corresponded to the predicted sizes. Digestion of the ITS region of *Candida* species by *Msp*I generated 2 bands for *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. krusei*, and 3 bands for *C. guilliermondii*. However, there were no recognition sites for this enzyme within the ITS region of *C. parapsilosis*, and its PCR and digestion products were the same size.

The RFLP pattern produced for each *Candida* species was completely specific so none of the species examined was mistaken for another (Fig 2, A). Identical patterns were seen for *C. albicans*, *C. albicans* var. *stellatoidea*, and *C. dubliniensis* (Fig2, B). None of the other yeasts tested, including *Cryptococcus neoformans*, *Saccharomyces cerevisiae*, and *Trichosporon asahii*, showed similar patterns (Fig2, B). The enzyme was also used for digestion of PCR products of 137 strains of *Candida* isolated from clinical specimens, including superficial, mucocutaneous, and deep seated specimens. Using this method, *C. albicans* was identified as the most common species (67.9%) followed by *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, and *C. krusei* (Table 3). In our study the results of PCR-RFLP tests for identification of the clinical isolates were completely identical to those obtained using CHROMagar *Candida*.

Discussion

Despite recent progress in the development of new molecular approaches for diagnosis of fungal infections, the goal of developing a simple, rapid, and cost effective test for diagnostic purposes remains elusive. In the present study, using the universal primers, ITS1 and ITS4, we amplified a fragment of 510−879 bp of the ITS1-5.8S-ITS2 rDNA region from genomic DNA of several strains of *Candida* species. Restriction fragment length polymorphism (RFLP) analysis of the PCR products with one enzyme allowed us to identify the 6 most medically important *Candida* species.

The ITS molecule contains several regions of highly conserved sequence useful for obtaining proper sequence alignments, but with sufficient sequence variability in other regions of the molecule that can serve as markers of species-specific restriction fragment length polymorphism (RFLP) [14]. This region has been used in part or completely by other investigators for species identification of some medically important fungi, particularly *Candida*[15, 16], *Aspergillus*[17], dermatophytes[18], *Trichosporon*[19], and *Malassezia*[20] species using various approaches, such as DNA probes, nested PCR, sequencing, and RFLP. Williams *et al.*[15] attempted to delineate medically important *Candida* species using restriction enzyme digestion (*Hae*III, *Dde*I, *Bfa*I) after amplification of the ITS1-ITS4 region, but their patterns were not suitable for identification, perhaps due to inaccessibility of related ITS1-ITS4 sequences. Maiwald *et al.*[21] introduced a method for presumptive differentiation of 12 clinically relevant yeasts to the species level by amplification of the small ribosomal subunit 18S-rRNA using six enzymes. Identification of *Candida* species by PCR-RFLP has also been applied recently by Deak and Pinto[22, 23]. While all of the mentioned studies discriminated *Candida* species using several restriction enzymes, we identified six species, which accounts for up to 95% of *Candida* infections, using only one enzyme.

In the present study, restriction digestion of the ITS amplification product with *Msp*I produced the predicted specific patterns for each species. Using this method, all standard stains were identified successfully. Moreover, the results of PCR-RFLP analysis of the clinical isolates examined were comparable with those obtained by CHROMagar *Candida* test. Unfortunately, digestion of *C. albicans* and *C. dubliniensis* with *Msp*I yielded similar patterns, and therefore additional enzymes for differentiation of these species are still required. Actually we have reported another PCR-restriction enzyme format for discrimination of these two *Candida* species[24].

We recommend this simple and easy-to-perform method for the identification of *Candida* isolates in the medical mycology laboratories.

Acknowledgments

We would like to thank Dr. M Fasihi and Mrs. Kh Omidi for their help.

References

1) Fridkin SK, Jarvis WR: Epidemiology of nosocomial fungal infections. Clin Microbiol Rev **9**: 499-511, 1996.
2) Jarvis WR: Epidemiology of nosocomial fungal infections, with emphasis on *Candida* species. Clin Infect Dis **20**: 1526-1530, 1995.
3) Wenzel RP: Nosocomial candidemia: risk factors and attributable mortality. Clin Infect Dis **20**: 1531-1534, 1995.
4) Wingard JR: Importance of *Candida* species other than *C. albicans* as pathogens in oncology patients. Clin Infect Dis **20**: 115-125, 1995.
5) Nguyen MH, Peacock JE, Morris AJ, Tanner DC, Nguyen ML, Snydman DR, Wagener MM, Rinaldi MG, Yu VL: The changing face of candidemia: emergence of non-*Candida albicans* species and antifungal resistance. Am J Med **100**: 617-623, 1996.
6) Reiss E, Tanaka K, Bruker G, Chazalet V, Coleman D, Debeaupuis JP, Hanazawa R, Latge JP, Lortholary J, Makimura K, Morrison CJ, Murayama SY, Naoe S, Paris S, Sarfati J, Shibuya K, Sullivan D, Uchida K, Yamaguchi H: Molecular diagnosis and epidemiology of fungal infections. Med Mycol **36**(Suppl 1): 249-257, 1998.
7) Yeo SF, Wong B: Current status of nonculture methods for diagnosis of invasive fungal infections. Clin Microbiol Rev **15**: 465-484, 2002.
8) Elie CM, Lott TJ, Reiss E, Morrison CJ: Rapid identification of *Candida* species with species-specific DNA probes. J Clin Microbiol **36**: 3260-3265, 1998.
9) Jordan JA: PCR identification of four medically important *Candida* species by using a single primer pair. J Clin Microbiol **32**: 2962-2967, 1994.
10) Haynes KA, Westerneng TJ: Rapid identification of *Candida albicans, C. glabrata, C. parapsilosis* and *C. krusei* by species-specific PCR of large subunit ribosomal DNA. J Med Microbiol **44**: 390-396, 1996.
11) Yamada Y, Makimura K, Uchida K, Yamaguchi H, Osumi M: Phylogenetic relationships among medically important yeasts based on sequences of mitochondrial large subunit ribosomal RNA gene. Mycoses **47**: 24-28, 2004.
12) Sugita T, Nishikawa A: Molecular taxonomy and identification of pathogenic fungi based on DNA sequence analysis. Nippon Ishinkin Gakkai Zasshi **45**: 55-58, 2004. (in Japanese)
13) Yamada Y, Makimura K, Mirhendi H, Ueda K, Nishiyama Y, Yamaguchi H, Osumi M: Comparison of different methods for extraction of mitochondrial DNA from human pathogenic yeasts. Jpn J Infect Dis **55**: 122-125, 2002.
14) Iwen PC, Hinrichs SH, Rupp ME: Utilization of the internal transcribed spacer regions as molecular targets to detect and identify human fungal pathogens. Med Mycol **40**: 87-109, 2002.
15) Williams DW, Wilson MJ, Lewis MA, Potts AJ: Identification of *Candida species* by PCR and restriction fragment length polymorphism analysis of intergenic spacer regions of ribosomal DNA. J Clin Microbiol **33**: 2476-2479, 1995.
16) Chen YC, Eisner JD, Kattar MM, Rassoulian-Barrett SL, LaFe K, Yarfitz SL, Limaye AP, Cookson BT: Identification of medically important yeasts using PCR-based detection of DNA sequence polymorphisms in the internal transcribed spacer 2 region of the rRNA genes. J Clin Microbiol **38**: 2302-2310, 2000.
17) Henry T, Iwen PC, Hinrichs SH: Identification of *Aspergillus* species using internal transcribed spacer regions 1 and 2. J Clin Microbiol **38**: 1510-1515, 2000.
18) Makimura K, Tamura Y, Mochizuki T, Hasegawa A, Tajiri Y, Hanazawa R, Uchida K, Saito H, Yamaguchi H: Phylogenetic classification and species identification of dermatophyte strains based on DNA sequences of nuclear ribosomal internal transcribed spacer 1 regions. J Clin Microbiol **37**: 920-924, 1999.
19) Sugita T, Nishikawa A, Ikeda R, Shinoda T: Identification of medically relevant *Trichosporon* species based on sequences of internal transcribed spacer regions and construction of a database for *Trichosporon* identification. J Clin Microbiol **37**: 1985-1993, 1999.
20) Makimura K, Tamura Y, Kudo M, Uchida K, Saito H, Yamaguchi H: Species identification and strain typing of *Malassezia* species stock strains and clinical isolates based on the DNA sequences of nuclear ribosomal internal transcribed spacer 1 region. J Med Microbiol **49**: 29-35, 2000.
21) Maiwald M, Kappe R, Sonntag HG: Rapid presumptive identification of medically relevant yeasts to the species level by polymerase chain reaction and restriction enzyme analysis. J Med Vet Mycol **32**: 115-122, 1994.
22) Deak R, Bodai L, Aarts HJ, Maraz A: Development of a novel, simple and rapid molecular identification system for clinical *Candida* species. Med Mycol **42**: 311-318, 2004.
23) Pinto PM, Resende MA, Koga-Ito CY, Ferreira JA, Tendler M: rDNA-RFLP identification of *Candida* species in immunocompromised and seriously diseased patients. Can J Microbiol **50**: 514-520, 2004.
24) Mirhendi H, Makimura K, Zomorodian K, Maeda N, Ohshima T, Yamaguchi H: Differentiation of *Candida albicans* and *Candida dubliniensis* using a single PCR-Restriction enzyme. Jpn J Infect Dis **58**: 235-257, 2005.

Jpn. J. Med. Mycol.
Vol. 47, 235−237, 2006
ISSN 0916−4804

Short Report

Development of Safety Culture Tube for Molds and Proposed Procedure for Collecting Conidia or Fixing Strains to Control Fungal Infection and Allergy

Koichi Makimura[1, 2], Takamasa Kaneko[1, 3], Masanobu Onozaki[1, 3], Miho Sugamata[1, 4], Hideyo Yamaguchi[1, 2], Shigeru Abe[1]

[1]Teikyo University Institute of Medical Mycology and [2]Genome Research Center, Graduate School of Medical Science, Teikyo University, 359 Otsuka, Hachioji, Tokyo 192-0395, Japan

[3]Kanto Chemical Co., Inc., 3-11-15 Nihonbashihoncho, Chuo-ku, Tokyo 103-0023, Japan

[4]Department of Sergical Pathology, Graduate School of Medicine, Toho University, 5-21-16 Omorinishi, Ota-ku, Tokyo 143-8541, Japan

[Received: 13, February 2006. Accepted: 9, May 2006]

Abstract

The conidia of filamentous fungi can be easily blown into the air and tend to be contaminants in the laboratory environment.

We developed a new "safety culture tube for fungi" to prevent biohazards and a procedure for collecting conidia for passage or fixing strains was proposed.

Key words: antifungal susceptibility testing, biohazard, conidia, DNA extraction, mold

Molds, especially *Aspergillus* species are not only life-threatening pathogenic fungi but are also major producers of mycotoxins[1]. It is essential to culture these fungi in microbiological laboratories in hospitals and public health institutes to analyze their features. However, the conidia of filamentous fungi can be easily blown into the air and tend to be contaminants in the laboratory environment. Moreover, such airborne conidia may cause allergy or infection laboratory members. This situation will be even more severe in the handling of highly pathogenic imported mycoses agents such as *Coccidioides immitis* or *Histoplasma capslatum*[2]. Therefore, it is necessary to develop means to harvest conidia or to fix cultures without contamination.

For the safe preparation of these fungi, we developed a new "safety culture tube for fungi" to prevent biohazards and a procedure for collecting conidia for passage or fixing strains was proposed.

Address correspondence to: Koichi Makimura
Teikyo University Institute of Medical Mycology
359 Otsuka, Hachioji, Tokyo 192-0395, Japan

Safety culture tube for fungi: The safety culture tube for fungi (Fig. 1A) is a ϕ 27×55 mm glass tube with a screw cap plugged with isobutene-isoprene rubber that can be penetrated with a needle to allow injection of liquids. Four ml of Yeast and Mold (YM) agar (Oxoid, Basingstoke, UK)[3] were prepared as slants in these tubes.

Fungal strain: As a representative airborne fungus, the *A. flavus* strain TIMM 2935, an aflatoxin producer and the second most common causative agent of aspergillosis in Japan, was used. Isolate was maintained on YM agar.

Using these culture tubes and this fungus, we assessed the proposed procedure for handling fungi for passage or fixing. Each preparation was performed three times over 40 open plates (5 lines and 8 columns) of YM agar in a safety cabinet adapted to the "cross-contamination prevention test," Japan Industrial Standard[4] to check for contamination. The procedure for collecting conidia for passage and antifungal susceptibility testing[5] was assessed as follows:

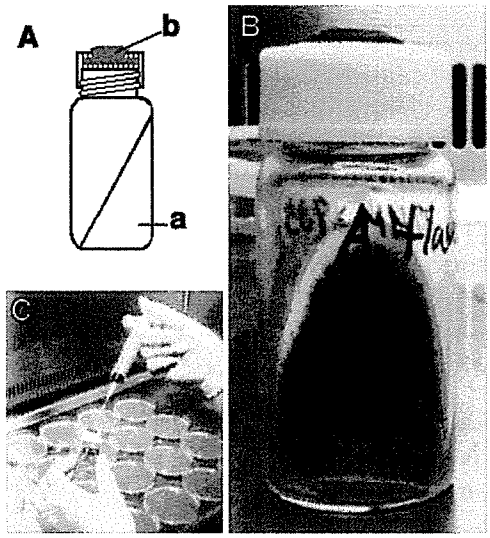

Fig. 1. Safety culture tube for fungi

A: Schema of the tube. a, agar slant; b, rubber plug.
B: *Aspergillus flavus* grew in the tube.
C: Conidia collecting solution or ethanol was injected over the culture plates to assess the ability of the tubes to prevent contamination.

(1) The strain or specimen was inoculated and grown in loosely capped culture tubes at 27°C for 7 days (Fig. 1B).
(2) The screw caps of the culture tubes were then closed tightly.
(3) Five m*l* of air was aspirated off using a syringe with a needle passing through the rubber plug, and 5 m*l* of conidia collecting solution (0.05% Tween 20, 0.85% saline) was then injected into the tube (Fig. 1C).
(4) After mixing, 1 m*l* aliquots of conidia suspension were harvested through the rubber plug.
(5) Suspensions were inoculated into fresh loosely capped culture tubes, then incubated at 27°C for 7 days or processed for antifungal susceptibility testing.

The cultures were fixed for DNA extraction as follows: Steps (1) and (2) were the same as described above. (3) Ten m*l* of air was aspirated off using a syringe with a needle inserted through the rubber plug, and 10 m*l* of 99% ethanol was injected into each tube and allowed to stand overnight at room temperature. (4) Mycelia were then picked up and processed for DNA extraction[6, 7]. The mycelia and the needle were also incubated in sterile tube with 50 m*l* of potato dextrose broth (Difco)[3]. All culture tubes and plates were incubated at 27°C for 7 days.

All 6 tests of 40 contamination control plates were culture-negative. Harvested conidia grew well in fresh tubes, similarly to the initial culture. However, no fungus grew from the needle or ethanol preparation. Fungal DNA was extracted from ethanol fixed mycelia and successfully amplified by polymerase chain reaction with fungal universal primers[6, 7] (data not shown).

These observations indicated that the process of passage from harvested conidia and fixing culture could be performed safely using the culture tubes without any contamination. Dry fungal conidia are hydrophobic and tend to become airborne contaminants. However, laboratory staff will be able to prepare fungal conidia both easily and safely if they are suspended with saline containing surfactant. Therefore, the culture tubes tested in the present study will be useful for routine laboratory work. As far as we know, the safety culture tube reported in this paper is a unique tool with which to prepare airborne fungal conidia without contamination or biohazard.

To test for antifungal susceptibility, the preparation of conidia suspension is required[5], and this tube meets this need. The conventional slide culture method[3] is essential for the identification of filamentous fungi. However, culture-based identification is time-consuming and sometimes dangerous, especially for highly pathogenic fungal strains. To resolve this problem, molecular biological identification systems[6, 7] have been reported. DNA was successfully extracted from the culture fixed in the culture tubes, therefore, the manner of fixing described here assures the safe manipulation of such biological agents.

This research was supported in part by Health and Labor Sciences Research Grants for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labor, and Welfare, Japan (KM).

References

1) Bennett JW, Klich M: Mycotoxins. Clin Microbiol Rev **16**: 497-516, 2003.
2) Kamei K, Sano A, Kikuchi K, Makimura K, Niimi, M, Suzuki K, Uehara Y, Okabe N, Nishimura K, Miyaji M: The trend of imported mycoses in Japan. J Infect Chemother **9**: 16-20, 2003.
3) Larone DH: Medically important Fungi-A guide to identification, 3rd edition, ASM Press, Washington, D.C, 1994.
4) Japan Industrial Standard (JIS). Class II biological safety cabinets, JIS K3800: 2000, 2000.
5) National Committee for Clinical Laboratory