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Microbiological characteristics of medically important *Trichosporon* species

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Abstract

Trichosporon species are opportunistic pathogens associated with a high mortality rate in immunocompromised patients. Disseminated trichosporonosis is uncommon but reports are increasing. In this study, using 16 stock clinical isolates of suspected *Trichosporon* species and 4 known *Trichosporon* strains, we investigated the morphology, physio-biochemistry, molecular biology and antifungal susceptibility characteristics of these *Trichosporon* spp. and discovered that ITS sequence-based identification is a rapid and accurate identification alternative to most phenotypic or physio- biochemical methods. In vitro antifungal susceptibility tests showed high amphotericin B, itraconazole and terbinafine MIC value in these *Trichosporon* strains.

Key words: antifungal susceptibility, ITS sequencing, *Trichosporon*, trichosporonosis

Introduction

Over the past decade, the incidence of serious infections caused by opportunistic fungal pathogens has increased dramatically due to alterations in the immune status of patients [1]. Mycoses in severely immunocompromised patients due to yeasts belonging to genera other than *Candida* have become increasingly common. The genus *Trichosporon* is a case in point.

Trichosporon is a medically important genus that includes the causative agents of systemic mycoses, mucosal-associated and superficial infections. Since the 1970s, *Trichosporon* spp. were initially recognized as pathogens capable of causing invasive disease [2]. Disseminated infections by this genus in patients who are immunosuppressed in the setting of hematologic or solid organ malignancy or solid organ transplantation have been increasing [3–6]. Gueho et al. [7, 8] performed an extensive taxonomic revaluation of strains representing the full range of species recovered from humans, animals and environmental source. It was shown that the formerly used designation *Trichosporon beigelii* included several genetically different species. A total of 19 taxa were delineated within this genus.

The common species causing trichosporonosis are *T. asahii*, *T. asteroides*, *T. cutaneum*, *T. inkin*, *T. mucoides* and *T. ovoides* [9]. Among them, two species were associated with deep infections in humans: *T. asahii* with cases of hematogenous, disseminated infections and *T. mucoides* with CNS infections. *T. asteroids* and *T. cutaneum* are mostly involved in superficial infections. *T. inkin* and *T. ovoides* are associated with white piedra of the head and genital areas, respectively. Deep-seated infections always occur in the immunocompromised host, especially neutropenic patients. Often these are refractory to antifungal therapy [3, 10] and the prognosis for such patients is very poor.

Trichosporon spp. are characterized by the production of true hyphae, pseudohyphae, arthroconidia and blastoconidia. Current conventional clinical identification of *Trichosporon* spp. mainly depends on cell and colony morphology and biochemical characteristics. This approach usually requires the technician to have extensive experience, and moreover, is time-consuming and not always fully discriminative.

In this study, using 16 clinical stocks of suspected *Trichosporon* spp. and four known *Trichosporon* strains, we systematically investigated the morphology, physio-biochemistry, molecular biology and antifungal susceptibility characteristics. These results will provide reference for clinic diagnosis and treatment of *Trichosporon* spp. infections as well as experience on how to rapidly and accurately identify these organisms.

Materials and methods

Strains

Sixteen stock clinical isolates, which were suspected *Trichosporon* spp. because of producing arthroconidia, and four known *Trichosporon* strains: two *T. asahii* (CBS 2479) and an environmental isolate; *T. cutaneum* (ATCC 28592) and *T. inkin* (UTHSCSA) were used in this study. All the clinical isolates were obtained from the culture collection of the Medical Mycology Research Center, Peking University, The First Hospital, PR China. The sources of strains used were listed in Table 1.

Cultural conditions

Colony morphology was observed on Petri dishes containing: Potato dextrose agar (PDA), Sabouraud dextrose agar (SDA), Brain heart infusion agar (BHIA), Oatmeal agar (OA), Corn meal agar (CMA), Czapek dox agar (CDA) and PDA + Yeast extract medium. All cultures were incubated at 28 °C and 35 °C and read after 10 days. All media were purchased from Difco Co. Subcultures for morphological studies were done on PDA and incubated at 28 °C for six days.

Carbohydrate assimilation test

Use API 20 C AUX system to identify the isolates to species.

In vitro *antifungal susceptibility testing*

The MICs of all isolates to antifungal agents were determined using the National Committee for Clinical Laboratory Standards (NCCLS) M-27A microdilution technique [11]. Approximately 1 ml of 0.85% saline containing conidia and hyphae grown on SDA for five days at 28 °C were dispersed with a vortex mixer. The optical densities of the conidial suspensions were adjusted with RPMI 1640 broth (Sigma) to approximately 1×10^3 ml^{-1}. Concentrated (2×) antifungal solutions were prepared in RPMI 1640 broth, and a serial dilution series was prepared in a 96-well microtiter plate. Final drug concentrations in the microtiter plates ranged from 64 to 0.064 μg ml^{-1} for fluconazole (FLU) and from 16 to 0.016 μg ml^{-1} for amphotericin B (AMB), terbinafine (TERB) and itraconazole (ITRA). The plates were incubated at 35 °C and the MICs were recorded after 48 h incubation (except *T. cutaneum* was incubated at 28 °C). The visually determined MIC was defined as the lowest drug concentration at which there was complete absence of growth (MIC-0) or a prominent growth reduction (MIC-2) in comparison with that of controls.

Table 1. Fungus isolates and species identification result based on API 20 C AUX system

BMU* strain No.	API code	Species	Source
00242	2545775	*T.inkin*	Blood UTHSCSA
00277	6745777	CNI	ATCC-28592
01463	2744734	*T.asahii*	CBS 2479 type strain
00563	2744775	*T.asahii*	Environmental isolate
01373	2744775	*T.asahii*	Clinical isolate
02746	2744775	*T.asahii*	Clinical isolate
02751	2744775	*T.asahii*	Clinical isolate
00903	2744734	*T.asahii*	Clinical isolate
00706	2744775	*T.asahii*	Clinical isolate
01431	2744775	*T.asahii*	Clinical isolate
02466	2744735	*T.asahii*	Clinical isolate
02744	2767776	CNI	Clinical isolate
02790	6000000	CNI	Clinical isolate
01135	6000000	CNI	Clinical isolate
02992	6000000	CNI	Clinical isolate
02993	6000000	CNI	Clinical isolate
02994	6000000	CNI	Clinical isolate
03067	6743775	CNI	Clinical isolate
03068	2540775	CNI	Clinical isolate
03069	2540775	CNI	Clinical isolate

CNI, Could not be identified through API 20 C AUX system.
*BMU: Beijing Medical University.

Genotypic analysis

Strains were inoculated onto PDA slants for five days at 28 °C. Fungal DNA isolation methods were adopted as described previously [12]. Approximately 2–3 gm of the fungus were scraped from the slant and added to 600 μl DNA extract buffer (100 mM Tris–HCl, Ph 9.0, 40 mM EDTA), 300 μl benzyl chloride, and 60 μl sodium dodecyl sulfate. Suspensions were then incubated in a 55 °C water bath for 1 h and vortexed every 10 min. Then, 60 μl 3M sodium acetate was added and the tube was kept in ice for 20 min. After centrifugation at 3500×*g* for 10 min, the supernatant was extracted with phenol-choroform (1:1). DNA was precipitated with isopropanol (1:1). After washing with 70% ethanol, the DNA pellet was resuspended in TE (Tris and EDTA) buffer.

The fragments containing the internal transcribed spacer 1 (ITS1) and ITS2 and the intervening 5.8s ribosome DNA (rDNA) region were amplified with previously published panfungal primers [13], primer ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′). The PCR reaction was performed in a final reaction mixture (50 μl) containing 20 pM of each primer; 200 mM (each) dATP, dGTP, dCTP and dTTP; 0.5 μl of Taq DNA polymerase; 5 μl of 10×PCR buffer (100 mM Tris–HCl, 20 mM $MgCl_2$, 500 mM KCl, 1.0% Triton X-100) and 1 μl of sample DNA. The amplification was performed in a PCR machine with the following cycling parameters: 95 °C for 5 min, followed by 30 cycle of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. The PCR products were purified and sequenced (ABI 3700 sequencer, USA) from both directions using one of the PCR primers as the sequencing primer. The nucleiotide–nucleiotide BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) was used to query the National Center for Biotechnology Information Genebank nucleotide database for homologous sequences. The sequences were aligned with Clustal procedure (http://ebi.ac.uk/Clustal).

Results

Based upon micro and macroscopic characteristics and other identification methods including the API 20C AUX system and ITS sequencing, nine of the 20 isolates were identified as *T. asahii*; one was *T. inkin*; one was *T. cutanum*; one was *T. dermatis*; three were *T. domesticum*; five were *Geotrichum capitatus*.

Morphology (Figure 1)

T. asahii was the most common species among the clinic isolates. The shape of the colony varied dependent on medium and temperature. Generally, after 10 days at 28 °C and 35 °C, the colonies were 16–22 mm in diameter, white, farinose at the center, with a wide, dry, often finely zonate margin with deep transverse fissures. Arthroconidia were abundant, rectangular, and had rounded ends.

T. cutaneum colonies were cream-colored, cerebriform, with broad, moist, glistening marginal zones. After 10 days at 28 °C, the colonies were 15–20 mm in diameter. Cultures generally consisted of disarticulating hyphae, but could revert to yeast forms with subspheroidal budding cells. Arthroconidia were regular, with lateral, clavate blastoconidia. *T. cutaneum* did not grow at 35 °C.

T. inkin colonies were also finely cerebriform, often cracking the media, with a white, farinose covering; a marginal zone was absent. After 10 days at 28 °C and 35 °C, the colonies were 16–20 mm in diameter. Appressoria were present. Hyphae disarticulated into rectangular anthroconidia.

T. domesticum grew more quickly at 28 °C than 35 °C. After 10 days at 28 °C and 35 °C, the colonies were cream to yellow, butyrous to membranous and 10–16 mm in diameter. Cultures generally consisted of septate hyphae with arthroconidia; vegetative reproduction was by splitting and budding.

There were no distinct differences between colony morphology of *G. capitatus* and *Trichosporon* species. *G. capitatus* colonies were 15–20 mm in diameter after 10 days at 28 °C and 35 °C. The colonies were usually glassy, later funiculose with a smooth expanding zone. Hyphae were branched at acute angles, often somewhat penicillate, conidia were clavate, with a truncate base. Arthroconidia were also common. No blastospores were seen.

Recently, *T. dermatis* was separated from *Cryptoccocus humicolus* because the molecular sequence analysis of the LSU rRNA suggests that *C. humicolus* is related to members of the genus

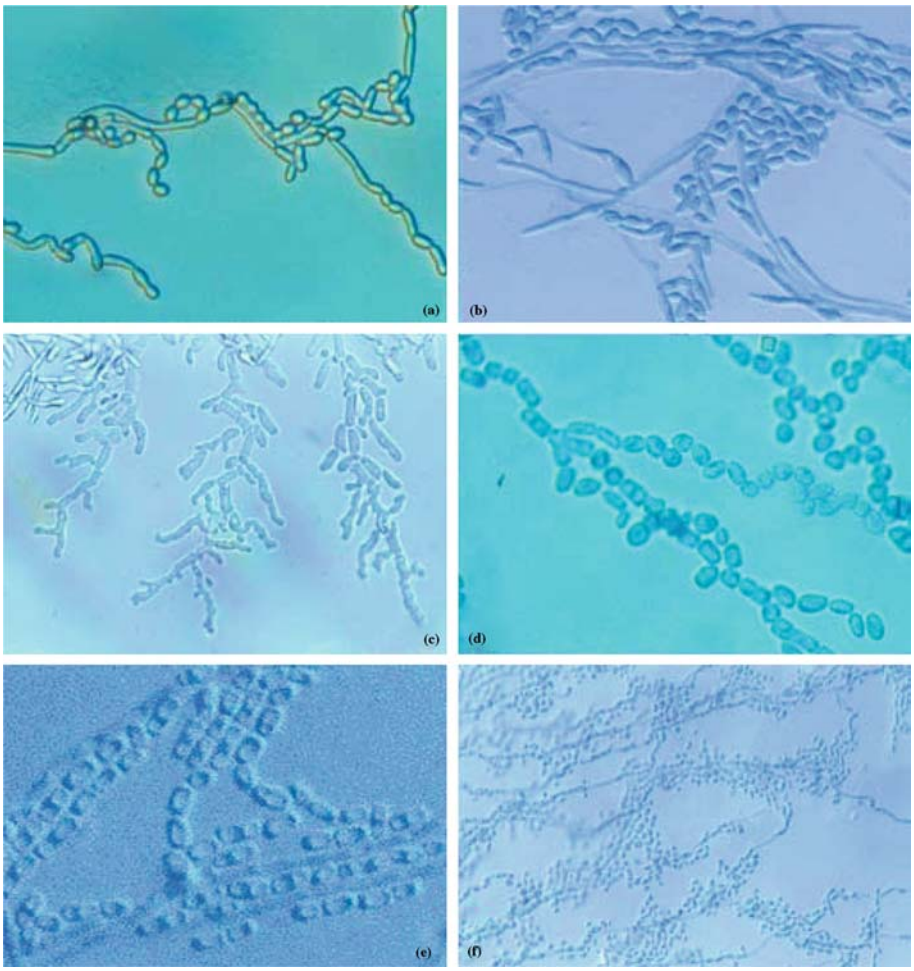

Figure 1. The colony and microscopic morphology of all species identified. Microscopic morphology of different species growing on PDA for six days. (a) *T. asahii*, (b) *T. cutaneum*, (c) *T. inkin*, (d) *T. domesticum*, (e) *G. capitatus* and (f) *T. dermatis* is also shown.

Trichosporon [14]. After 10 days at 28 °C and 35 °C, the colonies were 20–24 mm in diameter, the colony had a fuzzy appearance due to the presence of tufts of aerial hyphae. After growth for six days at 28 °C on PDA, pseudohyphae and true hyphae were abundantly formed. Hyphae and pseudohyphae were often wavy in appearance. Branching was characteristically at narrow angles resulting in branches that were almost parallel to the main axes. The ovoidal blastoconidia could form laterally on small terminal hyphal projections.

Carbohydrate assimilation test

Using the API 20 C AUX system, *T. asahii* and *T. inkin* could be easily identified**.** However, for the other species, the API 20 C AUX system was of little value and had to be supplemented with other methods such as growth at various temperatures and DNA sequence analyses. The results of the API are presented in Table 1.

In vitro *antifungal susceptibility testing*

For all species, the mean values of susceptibility testing are shown in Table 2. AMB MICs for the five species ranged from 1–16 μg/ml in vitro and all isolates were resistant to fungicidal activity of amphotericin B. FLU MICs were relatively low ranging from 1–32 μg/ml in vitro. Most strains exhibited high itraconazole and TERB MICs in vitro (MIC ranges: ITRA: 0.25–4 μg/ml; TERB: 4–16 μg/ml). Only these above-mentioned known

Table 2. Antifungal susceptibility results for all isolates

Drug		*T.asahii*	*T.cutanum*	*T.inkin*	*T.dermatis*	*G.capitatus*	*T.domesticum*
Fluconazole	MIC	2–16	32	4	1	8–32	4–16
	GM	10.3	–	–	–	20.8	14.6
Itraconazole	MIC	0.5–2	1	1	0.25	0.5–4	2–4
	GM	1.4	–	–	–	2.5	2.6
Amphotericin B	MIC	8–16	16	1	4	4–16	8–16
	GM	10.6	–	–	–	10.4	12.6
Terbinafine	MIC	4–16	1	1	2	4–16	4–8
	GM	12.6	–	–	–	10.4	5.2

strains of *T. asahii* and *T. cutaneum* were sensitive to itraconazole.

Sequence analysis

Using DNA analysis methods, the ITS-PCR fragment could be amplified easily from all the isolates. Figure 2 shows the nucleotide sequence of ITS1 and ITS2 regions of selected (i.e., non duplication of species) isolates. Although some consensus base sequences could be seen within all isolates, there are more similar in ITS-sequence and ITS-length between *trichosporon* species. The ITS sequence of each strain were compared to those available in the GenBank to identify species.

Discussion

Currently, conventional clinical laboratory procedures to identify *Trichosporon* spp. are inadequate. All of these fungi form arthroconidia but there are other fungi that also produce arthroconidia. This characteristic is of little value in identification to the species level.

Another commonly used identification method is the carbohydrate assimilation test, which measures the ability of a fungus to utilize a specific carbohydrate as the sole source of carbon in the presence of oxygen. The API 20C AUX system is a commercial kit that uses this method to help identify fungal species. To *Trichosporon* spp., this information is not very helpful in identification since it could does not differentiate most of the species. Temperature studies are also an important parameter to assist with *Trichosporon* spp. identification. *T. cutaneum* will not grow at 35 °C, perhaps why it occurs mostly as an agent of superficial mycoses of warm-blooded animals, including man. *T. loubieri* has good growth at 42 °C.

In addition to the above-mentioned methods, there are other identification parameters such as the urease test, phenol oxidase test and nitrate assimilation test that assist in the identification of fungi to genus and species levels. How to rapidly identify *Trichosporon* species by choosing various effective methods requires technicians with extensive experience. Commonly, a few laboratories will specialize in one of these techniques.

Ribosomal DNA (rDNA) has been widely utilized for molecular systematics and the identification of microorganisms. Molecular sequencing the entire ITS1-5.8s-ITS2 rDNA region can efficiently and effectively identify fungi to species level and have been commonly used to identify pathogenic fungi. Our results also suggest that a sequence-based identification method is a rapid, more accurate and effective alternative than most phenotypic or physio-biochemical methods. This viewpoint is also held by some other investigators [15]. Except ITS sequencing, Sugita T et al. demonstrated that the divergence of intergenic spacer (IGS) region, which is located between the 26S and 5S rRNA genes, has been greater than that of ITS region. Thus, IGS sequence analysis can also be ultilized to differentiate closely related *Trichosporon* species [16]. Although DNA sequencing give more definitive results but is not achievable for many laboratories. Thus, a more practical approach for routine use is needed to identify the clinical *Trichosporon* strains to species level.

Five of our 20 isolates were identifed as *Geotrichum capitatum*, also known as *Dipodascus capitatus* (the telemorph). Other names for this organism include *Trichosporon capitatum*, *Blasto-*

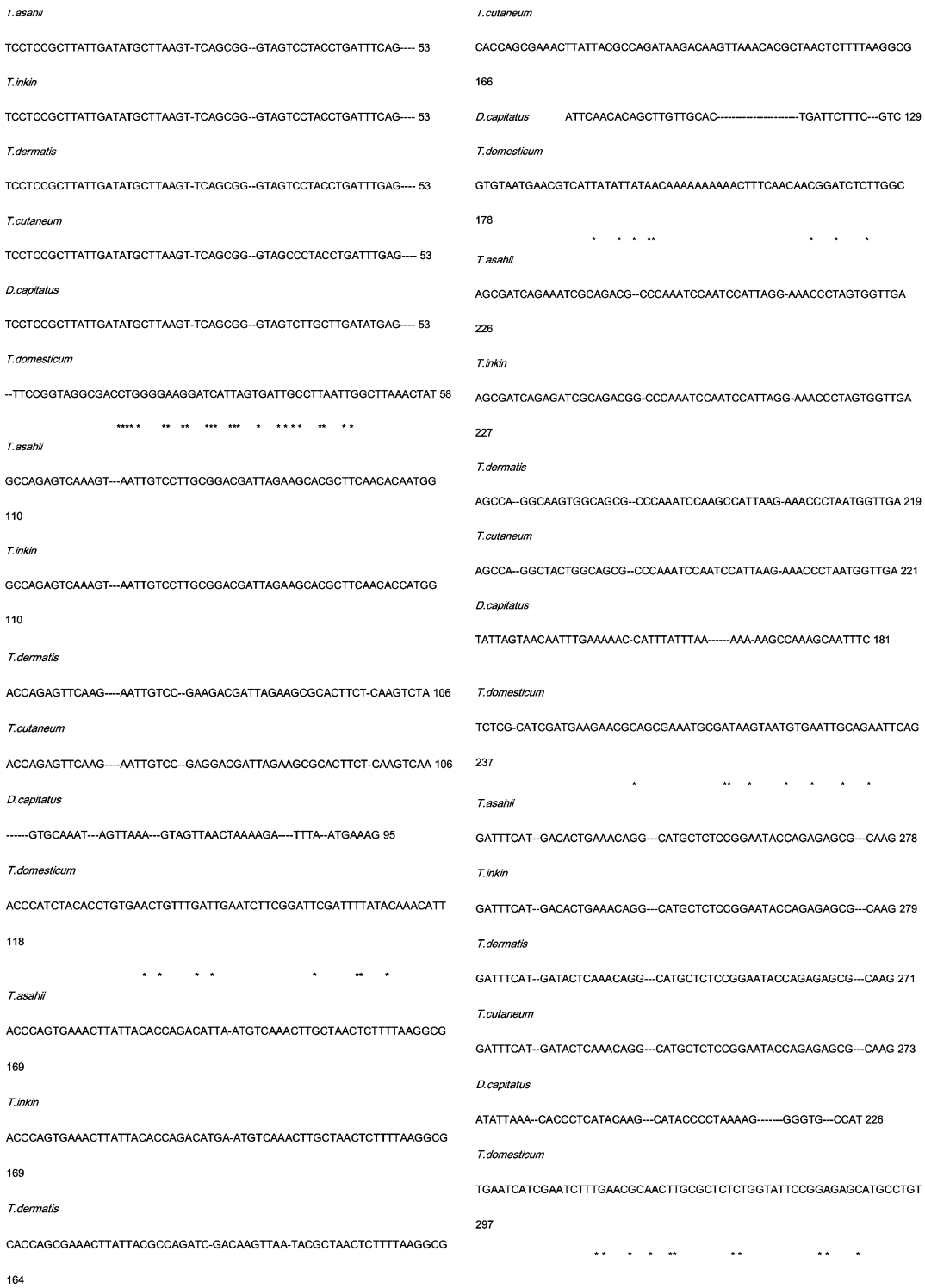

Figure 2. Alignment of the isolates ITS sequences. Alignment of the *Trichosporon* spp. and *Geotrichum capitatus* ITS 1, ITS2 and the intervening 5.8s ribosome DNA (rDNA) sequence. Asterisks are used when the nucleotide at a particular position is identical in five species. Dashes represent deletions necessary for alignment.

T.asahii

CTGCGT-TCA--AAGATTCGATGATTCACTGAATTCTGCAATTCACATT---ACTTATCG 332

T.inkin

CTGCGT-TCA--AAGATTCGATGATTCACTGAATTCTGCAATTCACATT---ACTTATCA 333

T.dermatis

TTGCGT-TCA--AAGATTCGATGATTCACTGAATTCTGCAATTCACATT---ACTTATCG 325

T.cutaneum

TTGCGT-TCA--AAGATTCGATGATTCACTGAATTCTGCAATTCACATT---ACTTATCG 327

D.capitatus

GTGCGT-TCA--AGAATT-GATGATTCAC-------TGCAATACACATC---ACATTTCG 272

T.domesticum

TTGAGTGTCATGAAATCTCAACCATTAGGGTTTCTTAATGGCTTGGATTTGGAGGTTTGC

357

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T.asahii

CAATTCGCTGCGTTCTTCATCGATGCGAGAGCCAAGAGATCCGTTGTTG---AAAGTTTT

389

T.inkin

CAATTCGCTGCGTTCTTCATCGATGCGAGAGCCAAGAGATCCGTTGTTG---AAAGTTTT

390

T.dermatis

CATTTCGCTGCGTTCTTCATCGATGCGAGAGCCAAGAGATCCGTTGTTG---AAAGTTAT

382

T.cutaneum

CATTTCGCTGCGTTCTTCATCGATGCGAGAGCCAAGAGATCCGTTGTTG---AAAGTTAT

384

D.capitatus

CAATTCGCTGCGTTCTTCATCGATGCGAGAGCCAAGAGATCCGTTGTTG---AAAGTTTT

329

T.domesticum

CAGTCTGACTGGCTCCTCTTAAAAGAGTTAGCAAGTTGAACTATTGCTATCTGGCGTAAT

417

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T.asahii

A--TTTTGTTATAATAAAAC-----GACGTTCATTACACATTGTTTGTAAAAATACTCGA 442

T.inkin

A--TTTTGTTATAATAAAAC-----GACGTTCATTACACATTGTTTGTAAAAACACTCGA 443

T.dermatis

---TTTTGTTATAATAACAT-----GACGTTCATTACACAATGTTTGTAAAAGTAATTGA 434

T.cutaneum

---TTTTGTTATAATAACAT-----GACGTTCATTACACAATGTTTGTAAAATCAATTGA 436

D.capitatus

A--TTTTGTTATAATAAAAC-----GACGTTCATTACACATTGTTTGTAAAAATACTCGA 382

T.domesticum

AAGTTTCGCTGGAATGGTATTGTGAAGCGTGCTTCTAATCGTCTTCGGACAATTTTTTGA

477

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T.asahii

CT--TGCGTCAAGTAGTAGCACAGTTCACAGGTGTAAGTGGATATAGTTATAAGCCTATA

500

T.inkin

CT--TGCGTCAAGTGGTAGAACAGTTCACAGGTGTATGTGGATATAGTTA-ACGC----- 495

T.dermatis

CC--GAAGTCAATCA-----ACAGTTCACAGGTGTAGATGGATATAGTTTAACGCTCAAA 487

T.cutaneum

CC--GAAGTCAATCA-----ACAGTTCACAGGTGTAGATGGATGTAGTT-AACGCTCAGA 488

D.capitatus

CT--TGCGTCAAGTAGTAGAACAGTTCACAGGTGTAAGTGGATATAGTTATAAGCCTATA

440

T.domesticum

CTCTGGCCTCAAATCA-GGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGG

536

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T.asahii AAGGCAATCACTAATGATCCTTCCGCAGGTTCACCTACGGA 541

T.inkin ----------

T.dermatis GAGCAATTCACTAATGATCCTTCCGCAGGTTCACCTACGGA 528

T.cutaneum GAGCAATTCACTAATGATCCTTCCGCAGGTTCACCTACGGA 529

D.capitatus AAGGCAATCACTAATGATCCTTCCGCAGGTTCACCTACGGA 481

T.domesticum AA---------- 538

Figure 2. Continued.

schizomyces capitatus and *Blastoschizomyces pseudotrichosporon* [17]. This organism has recently been recognized as an emerging cause of invasive fungal disease in leukemia patients and patients with endocarditis [18, 19]. This species belongs to *trichosporon* before and on the basis of morphologic features, *Geotrichum capitatum* can be difficult to separate from *Trichosporon* spp. In some situations, arthroconidia and blastoconidia can be observed in the *Trichosporon* species and no blastoconidium production in *G. capitatum*. In addition, *G. capitatum* may be separated from *Trichosporon* spp. by the lack of urease. These may be significant since both were not identifiable with the API system. We also were able to differentiate them effectively with ITS sequencing.

Despite the increased frequency of trichosporonosis, data on the antifungal susceptibilities of *Trichosporon* spp. are limited. Meanwhile, the increase in these pathogens associated with serious fungal infections has not been matched by a corresponding increase in the number of antifungal agents available for their treatment. In fact, clinical treatment failures with amphotericin B or fluconazole or a combination of the two have been reported [3, 10, 20]. In this study, we tried to determine the in vitro activities of various antifungals against *Trichosporon* spp. and *G. capitatum* using the NCCLS M27-A broth microdilution method even though the recommendations for in vitro testing of this fungus are not included in the guideline.

AMB MICs were relatively high for all the isolates. This has profound significance clinically since we have also observed (unpublished observations) that these drugs are of limited (or of no) value in the treatment of our patients. The results also confirm previous observations [21–23] suggesting that AMB treatment alone for trichosporonosis is not recommended, especially when host defenses are compromised. FLU MICs were not high, but the in vitro observation has not correlated well with good clinical outcomes [24]. Surprisingly we found that most of our clinical isolates had relatively high MICs. These observations had some differences with previous findings [24]. Because the known strain *T. asahii* was sensitive to ITRA, we suspect that the resistance of clinical strains might be correlated with over use of antifungals agents. This must be extended to more strains and patients. Now M27-A2 document have replaced M-27A after we have finished this experiments. Experience of in vitro antifungal susceptibility testing against *Trichosporon* spp. with this new method is needed. Also studies for in vivo correlation of these findings should be investigated further.

Among the new antifungal drugs, some new triazole derivatives, such as voriconazole, posaconazole and ravuconazole appear to be more active against *Trichosporon* spp. than the older line antifungaldrugs [23]. But the echinocandins, inhibitors of cell wall synthesis, had no activity against *Trichosporon* species [25, 26]. A possible approach to overcoming antifungal drug resistance and high mortality rates seen in severe trichosporonosis is to combine two or three classes of antifungals, especially if the drugs have different mechanisms of action.

In conclusion, comparative sequence analysis of medically relevant *Trichosporon* species can be readily identified by their ITS sequence. Difference from conventional clinical identification method of *Trichosporon* spp. which mainly depends on cell and colony morphology and biochemical characteristics, this is a more convenient and effective identification system.

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