Characterization of *Trichosporon* species isolated from clinical specimens in Kuwait

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Invasive trichosporonosis is an emerging infection of severely immunocompromised patients. It is principally caused by *Trichosporon asahii*, although some other species of the genus have also been implicated in the aetiology. In this work, 29 clinical isolates of *Trichosporon* species recovered from 29 different patients over a 6-year period were studied for phenotypic and genotypic characteristics. Two morphotypes were recognized on the basis of colony characteristics. The colonies of 25 isolates appeared flat and centrally wrinkled, while the other four isolates appeared dry and irregularly folded or verrucosed. Based on substrate assimilation profiles, all 29 isolates were identified as *T. asahii* using the Vitek 2 system. However, PCR amplification of rRNA gene sequences identified only 25 of the 29 isolates as *T. asahii*. The identity of the remaining four isolates was established as *Trichosporon asteroides* by direct DNA sequencing of the internally transcribed spacer (ITS)-1 and ITS-2 regions in the rRNA gene fragment amplified by PCR using panfungal primers. Fingerprinting carried out by randomly amplified polymorphic DNA analysis showed genotypic heterogeneity among the 25 *T. asahii* and four *T. asteroides* isolates. These data suggest that *T. asahii* is the major species associated with clinical specimens in Kuwait.

Received 4 December 2004

Accepted 16 March 2005

INTRODUCTION

The genus *Trichosporon* Behrend includes anamorphic yeasts with distinct morphological characteristics of budding yeast cells and true mycelia that disarticulate to form arthroconidia. In 1992, Gueho *et al.* (1992) revised the genus *Trichosporon* using the characteristics of morphology, ultrastructure, physiology, the ubiquinone system, DNA G+C content, DNA–DNA reassociation and 26S rRNA gene partial sequences. Based on a study of 101 strains isolated from diverse sources, 19 taxa were distinguished. Within the taxa isolated from humans, a distinction was made between those isolated from systemic infections and those that caused pubic or non-pubic white piedra. In a subsequent study, Sugita *et al.* (1995) investigated ten clinical isolates of *Trichosporon cutaneum* originating from deep-seated and mucosa-associated infections by use of a DNA–DNA reassociation technique and identified nine of the isolates as *Trichosporon asahii* and one as *Trichosporon ovoides*. These findings suggested that *T. cutaneum* is a heterogeneous species and that the causative agents of trichosporonosis exist in four or more species. Since the publication of major monographs on yeast taxonomy, each containing 19 *Tri-*

chosporon species, 15 additional species have been described (Fell & Scorzetti, 2004; Middelhoven *et al.*, 2004). These new species have been identified on the basis of DNA sequence analysis of the internally transcribed spacer (ITS) regions and/or the D1/D2 region of the large subunit (26S) rRNA gene. In addition, assimilation of some new substrates, not traditionally used in yeast taxonomy, was also employed for delineating some of these species (Middelhoven *et al.*, 2004). The genus *Trichosporon* now comprises 34 species/varieties (Fell & Scorzetti, 2004; Middelhoven *et al.*, 2004), seven of which, namely *T. asahii*, *Trichosporon mucoides*, *Trichosporon asteroides*, *T. cutaneum*, *Trichosporon inkin*, *T. ovoides* and *Trichosporon loubieri*, are known human pathogens causing disseminated or superficial infections (de Hoog *et al.*, 2000; Middelhoven, 2003; Marty *et al.*, 2003; Padhye *et al.*, 2003). Additionally, *T. asahii*, *T. mucoides*, *T. ovoides*, *Trichosporon montevidense* and *Trichosporon domesticum* have also been implicated in summer-type hypersensitivity pneumonitis (Gueho *et al.*, 1994; Nishiura *et al.*, 1997; Sugita *et al.*, 1998a).

There has been no study on phenotypic and genotypic characterization of *Trichosporon* species from the Middle East. In this communication, we report, for the first time, results of morphological, biochemical and molecular identification of 29 consecutive isolates of *Trichosporon* species obtained over a 6-year period from 29 different patients in Kuwait.

Abbreviations: ITS, internally transcribed spacer; RAPD, random amplification of polymorphic DNA.

The GenBank/EMBL/DBJ accession numbers for genes from *Trichosporon asahii* strains are AJ864866 and AJ864867, and for *Trichosporon asteroides* strains are AJ864868–AJ864871.

METHODS

Reference strains. The reference strains of *Trichosporon* species used in this study were obtained from the Centraalbureau voor Schimmelf-cultures (CBS), Utrecht, The Netherlands. The following strains were used: *T. asahii* var. *asahii* CBS 2479, *T. asahii* var. *asahii* CBS 2530, *T. asahii* var. *faecale* CBS 4828, *T. asteroides* CBS 2481, *T. inkin* CBS 5585, *T. mucoides* CBS 7625 and *Trichosporon jirovecii* CBS 6864. The strains were grown on Sabouraud dextrose agar (SDA) (Difco Labora-tories). Other reference strains used in this study included *Candida albicans* (ATCC 76615), *Candida dubliniensis* (type strain CD 36), *Candida glabrata* (ATCC 15545), *Candida parapsilosis* (ATCC 10233), *Candida tropicalis* (ATCC 750), *Aspergillus fumigatus* (CBS 113.26), *Aspergillus terreus* (CBS 118.27), *Aspergillus flavus* (CBS 113.32), *Fusarium solani* (ATCC 36031), *Fusarium oxysporum* (CBS 109898), *Cryptococcus neoformans* (ATCC 90112), *Mycobacterium tuberculosis* H₃₇Rv and *Escherichia coli* BL21.

Isolation and identification of *Trichosporon* species. A total of 4007 clinical specimens were processed over a 6-year period (1997–2002) for the isolation of pathogenic fungi according to standard procedures (McGinnis, 1994) (Table 1). The clinical specimens were cultured on SDA supplemented with chloramphenicol (50 mg l⁻¹). Blood specimens were cultured either by BACTEC 9240 (Becton Dickinson) or the Isostat lysis centrifugation system (Wampole Laboratories). Culture plates were incubated at 28 °C and observed daily for growth for up to 1 week. Colonies appearing as yeast-like in consistency were examined as a wet mount for microscopic character-istics. A total of 29 isolates showing budding yeast cells, hyphae and arthroconidia were provisionally identified as *Trichosporon* species (Table 1).

Study of morphologic characteristics and biochemical profile. The reference strains and clinical isolates were studied for colonial and microscopic characteristics on SDA and malt extract agar (MEA), respectively. Apart from rate of growth, the study of colony character-istics included texture, topography and colour. A small inoculum from an isolated colony of reference strains and test isolates was inoculated in the centre of the SDA plate. The plates were incubated at 28 °C and examined for growth for up to 10 days. Microscopic morphology was studied by the Dalmau technique (McGinnis, 1994). The slides were incubated in a moist chamber for 24–48 h and examined under high-power magnification for the formation of hyphae, budding yeast cells, arthroconidia, apressoria and sarcinae.

The reference strains of *T. asahii*, *T. asteroides*, *T. inkin*, *T. mucoides* and all the clinical isolates were studied for substrate assimilation profiles employing the Vitek 2 yeast identification system performed as recommended by the manufacturer. Each test isolate was freshly subcultured by streaking. The yeast suspension was automatically inoculated into a Vitek 2 ID-YST card and incubated at 35 °C. Readings

were taken after 24 h incubation. The Vitek 2 ID-YST card has a database for 46 substrates and is programmed to identify only three *Trichosporon* species, namely *T. asahii*, *T. inkin* and *T. mucoides*.

PCR-based detection of *Trichosporon* species DNA. Genomic DNA from the reference and clinical *Trichosporon* species, as well as from other fungal and bacterial organisms, was isolated as described previously (Sugita *et al.*, 1995; Ahmad *et al.*, 2002). DNA samples were stored at –20 °C until use. To design primers that would specifically amplify *T. asahii* or *T. mucoides* DNA, the sequences of the ITS region (including the ITS-1 region, the 5-8S rRNA gene and the ITS-2 region), as well as the DNA sequences at the 3' end of the 18S and the 5' end of the 26S rRNA genes from several *Trichosporon* species, were aligned using CLUSTAL W (<http://www.ebi.ac.uk/clustalw>). Based on this alignment, two primer pairs (TASF+TASR and TMF+TMR) were designed for the detection of *T. asahii* and *T. mucoides* DNA, respectively, by PCR. The sequences of these primers were: TASF, 5'-GGATCATTAGTGATTGC CTTTATA-3'; TASR, 5'-AGCACGCTTCAACACAATGGAC-3'; TMF, 5'-GGATCATTAGTGAATTGCTCTTTGA-3'; and TMR, 5'-TTAGAA GCGCACTTCTCAAGTCT-3'. The species specificity of the primers was confirmed by performing a BLAST search (Altschul *et al.*, 1990). The TASF primer is specific for *T. asahii* and has been described previously (Sugita *et al.*, 1998b, c), while the sequence of our TASR primer matched the sequence from five *Trichosporon* species, namely *T. asahii*, *T. asteroides*, *Trichosporon japonicum*, *T. ovoides* and *Trichosporon aquatile*. Furthermore, the DNA sequences of the TMF and TMR primers were found to be specific for four *Trichosporon* species, namely *T. mucoides*, *T. jirovecii*, *T. cutaneum* and *Trichosporon dermatis*. The clinical isolates that did not yield an amplified product by PCR with *T. asahii*-specific or *T. mucoides* primers were tested with the panfungal primers ITS1, ITS2, ITS3 and ITS4 (Ahmad *et al.*, 2002; Fujita *et al.*, 1995). The location of the TASF/TMF primers in the ITS-1 region and the TASR/TMR primers in the ITS-2 region, as well as the primer-binding locations of the panfungal primers ITS1, ITS2, ITS3 and ITS4 used for PCR amplifica-tion of rRNA gene fragments, are shown in Fig. 1. PCR amplification with *T. asahii*-specific, *T. mucoides* or panfungal primers was performed with genomic DNA prepared from reference or clinical isolates of *Trichosporon* species and the amplified products were resolved by agarose gel electrophoresis as described previously (Ahmad *et al.*, 2002).

DNA sequencing. To confirm the results obtained with assimilation patterns using the Vitek 2 system and PCR amplification of genomic DNA using *T. asahii*-specific primers, direct DNA sequencing of the ITS-1 and ITS-2 regions in the PCR-amplified products was performed on two clinical isolates identified as *T. asahii*. DNA sequencing was also performed for clinical isolates that were identified as *T. asahii* based on assimilation patterns by Vitek 2 but that did not yield an amplified product with TASF/TASR or TMF/TMR primer combinations in PCR. For this purpose, the amplified DNA obtained with the ITS1 and ITS4 primers was subjected to sequencing and both strands were sequenced

Table 1. Recovery of *Trichosporon* species isolates from clinical specimens in Kuwait during a 6-year period (1997–2002)

Year of isolation	No. of clinical specimens processed	No. of <i>Trichosporon</i> species isolated
1997	257	1
1998	735	6
1999	420	2
2000	598	7
2001	880	6
2002	1117	7

Fig. 1. The 18S rRNA gene, the ITS region (including ITS-1, the 5-8S rRNA gene and ITS-2) and the 26S rRNA gene showing the location of the *T. asahii*-specific primers (TASF and TASR), *T. mucoides*/*jirovecii* primers (TMF and TMR) and the panfungal primers ITS1, ITS2, ITS3 and ITS4 used for PCR amplification.

(Ahmad *et al.*, 2004). DNA sequencing data were analysed using BLAST (Altschul *et al.*, 1990).

Fingerprinting of *Trichosporon* species isolates by random amplification of polymorphic DNA (RAPD) analysis. Two short oligonucleotides were selected as single primers for fingerprinting of the 29 clinical *Trichosporon* species isolates by RAPD. The DNA sequences of these primers were GAC-1 (5'-GACGACGACGAC-3') and M13 (5'-GAGGGTGGCGGTTCT-3') (Sugita *et al.*, 2001a). The two RAPD assays were individually optimized (Ahmad *et al.*, 2003; Sugita *et al.*, 2001a). Amplifications in a total volume of 50 µl contained 5 µl 10× PCR buffer II (Perkin Elmer), 5 µl 25 mM MgCl₂, 200 µM dNTPs, 10 pmol GAC-1 or M13 primer, 2 U AmpliTaq DNA polymerase (Perkin Elmer) and 2 µl genomic DNA. For primer GAC-1, an initial denaturation at 95 °C for 3 min was followed by 40 cycles of 1 min at 95 °C, 30 s at 45 °C and 2.5 min at 72 °C, and one cycle of 10 min at 72 °C. For primer M13, the cycling parameters included an initial denaturation at 95 °C for 3 min, 40 cycles of 1 min at 95 °C, 30 s at 40 °C and 2.5 min at 72 °C, followed by one cycle of 72 °C for 10 min. Amplified products were separated by agarose gel electrophoresis and similarities and differences

among DNA fragment banding patterns were visualized and recorded (Ahmad *et al.*, 2003).

RESULTS

Of the 4007 clinical specimens processed during the study period (January 1997–December 2002), 29 isolates (K1–K29) of *Trichosporon* species originating from 29 different patients were obtained (Table 1). Information pertaining to the source of isolation and the underlying diseases of the patients yielding these isolates is provided in Table 2. The study of colony characteristics of the 29 clinical isolates on SDA revealed two distinct morphotypes: 25 isolates yielded cream-coloured colonies (18–34 mm diameter), which appeared flat with a farinose covering (Fig. 2a), while the remaining four isolates yielded smaller (19–24 mm diameter), irregularly folded or verrucosed colonies (Fig. 2b). Similar features were exhibited by the reference strains of

Table 2. Phenotypic and molecular characteristics of *Trichosporon* species isolates recovered from clinical specimens in Kuwait

Isolate no.	Clinical specimen	Underlying disease	Colony characteristics*	Species identification		
				Vitek 2 ID-YST	rRNA gene PCR	rRNA gene sequencing
K1	Urine	NA	Type 1	<i>T. asahii</i>	<i>T. asahii</i>	ND
K2	Urine	Kidney transplant	Type 1	<i>T. asahii</i>	<i>T. asahii</i>	ND
K3	Urine	Cancer	Type 1	<i>T. asahii</i>	<i>T. asahii</i>	ND
K4	Urine	Cancer	Type 1	<i>T. asahii</i>	<i>T. asahii</i>	ND
K5	Urine	Cancer	Type 1	<i>T. asahii</i>	<i>T. asahii</i>	ND
K6	Urine	Severe burns	Type 1	<i>T. asahii</i>	<i>T. asahii</i>	ND
K7	Urine	Hemi-colectomy	Type 1	<i>T. asahii</i>	<i>T. asahii</i>	ND
K8	Peritoneal fluid	Kidney failure	Type 1	<i>T. asahii</i>	<i>T. asahii</i>	ND
K9	Sputum	RTA	Type 1	<i>T. asahii</i>	<i>T. asahii</i>	ND
K10	Urine	NA	Type 1	<i>T. asahii</i>	<i>T. asahii</i>	ND
K11	Urine	NA	Type 1	<i>T. asahii</i>	<i>T. asahii</i>	ND
K12	Soft tissue	NA	Type 1	<i>T. asahii</i>	<i>T. asahii</i>	ND
K13	Urine	NA	Type 1	<i>T. asahii</i>	<i>T. asahii</i>	ND
K14	Urine	Low birth weight	Type 1	<i>T. asahii</i>	<i>T. asahii</i>	ND
K15	Catheter site	AML	Type 1	<i>T. asahii</i>	<i>T. asahii</i>	ND
K16	Sputum	Chest infection	Type 1	<i>T. asahii</i>	<i>T. asahii</i>	ND
K17	Urine	NA	Type 1	<i>T. asahii</i>	<i>T. asahii</i>	ND
K18	Urine	NA	Type 1	<i>T. asahii</i>	<i>T. asahii</i>	<i>T. asahii</i>
K19	Urine	Cancer	Type 1	<i>T. asahii</i>	<i>T. asahii</i>	<i>T. asahii</i>
K20	Blood	Cancer	Type 2	<i>T. asahii</i>	Unidentified	<i>T. asteroides</i>
K21	Blood	Kidney failure	Type 2	<i>T. asahii</i>	Unidentified	<i>T. asteroides</i>
K22	Blood	Cirrhosis	Type 1	<i>T. asahii</i>	<i>T. asahii</i>	ND
K23	Catheter tip	Low birth weight	Type 2	<i>T. asahii</i>	Unidentified	<i>T. asteroides</i>
K24	Urine	NA	Type 1	<i>T. asahii</i>	<i>T. asahii</i>	ND
K25	Urine	NA	Type 1	<i>T. asahii</i>	<i>T. asahii</i>	ND
K26	Urine	NA	Type 1	<i>T. asahii</i>	<i>T. asahii</i>	ND
K27	Urine	NA	Type 1	<i>T. asahii</i>	<i>T. asahii</i>	ND
K28	Urine	Kidney failure	Type 1	<i>T. asahii</i>	<i>T. asahii</i>	ND
K29	Blood	Pleural effusion	Type 2	<i>T. asahii</i>	Unidentified	<i>T. asteroides</i>

AML, acute myelocytic leukaemia; NA, not available; ND, not done; RTA, road traffic accident.

*Type 1 colonies initially appeared flat and centrally wrinkled with a farinose covering; type 2 colonies were dry and irregularly folded or verrucosed.

Fig. 2. Flat, centrally wrinkled colony of a clinical isolate of *T. asahii* (strain K17) (a) and folded (verrucosed) colony of *T. asteroides*, strain K20 (b) on SDA after 10 days at 28 °C.

T. asahii (CBS 2479) and *T. asteroides* (CBS 2481). Three of the clinical *T. asteroides* isolates also showed cracking of the agar. No discernible differences were observed in microscopic morphology. The assimilation profiles of the reference strains of *T. asahii* (CBS 2479, CBS 2530 and CBS 4828) and *T. asteroides* (CBS 2481) by the Vitek 2 system were not discriminatory. When the clinical isolates from Kuwait were subjected to identification by Vitek 2 ID-YST cards, all 29 isolates were identified as *T. asahii*. Of the 46 substrates included in the Vitek 2 ID-YST cards, 18 were assimilated by all the clinical isolates and eight were not, while 20 yielded variable results (data not shown), indicating the inability of the Vitek 2 identification system to discriminate among various *Trichosporon* species.

PCR performed with *T. asahii*-specific TASF and TASR primers yielded specific amplification of a DNA fragment (~430 bp) with genomic DNA from the reference cultures of two strains of *T. asahii* var. *asahii* (Fig. 3a, lanes 1 and 2) and from *T. asahii* var. *faecalis* (lane 3), but not from *T. asteroides* (lane 4), *T. inkin* (lane 5), *T. mucoides* (lane 6) or *T. jirovecii* (data not shown). Furthermore, PCR performed with *T. mucoides* primers (TMF and TMR) yielded specific amplification of a DNA fragment (~416 bp) with genomic DNA from reference cultures of *T. mucoides* (Fig. 3b, lane 5) and *T. jirovecii* (lane 6), but not from *T. asahii* var. *asahii*, *T.*

Fig. 3. Agarose gels of PCR products amplified from genomic DNA using the TASF and TASR primers (a) from *T. asahii* strains CBS 2479, CBS 2530 and CBS 4828 (lanes 1–3, respectively), *T. asteroides* (lane 4), *T. inkin* (lane 5) and *T. mucoides* (lane 6), and using the TMF and TMR primers (b) from *T. asahii* strains CBS 2479 and CBS 4828 (lanes 1 and 2, respectively), *T. asteroides* (lane 3), *T. inkin* (lane 4), *T. mucoides* (lane 5) and *T. jirovecii* (lane 6). Lane M, 100 bp DNA marker, with the positions of 100 and 600 bp fragments indicated.

asahii var. *faecalis*, *T. asteroides* and *T. inkin* (lanes 1–4, respectively). No amplification of a DNA fragment of any size was obtained when PCR was performed with *T. asahii*-specific or *T. mucoides* primers with genomic DNA isolated from reference cultures of *Candida albicans*, *Candida dubliniensis*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, *A. fumigatus*, *A. terreus*, *A. flavus*, *F. solani*, *F. oxysporum*, *Cryptococcus neoformans*, *M. tuberculosis* or *E. coli* (data not shown).

PCR amplification of genomic DNA with *T. asahii*-specific primers resulted in specific amplification of a single DNA fragment (~430 bp) from 25 clinical *Trichosporon* isolates only (Fig. 4, lanes K1–K19, K22, K24–K28), while the remaining four isolates did not yield an amplified product. None of the 29 isolates yielded an amplified product when PCR amplification was performed with the TMF and TMR primers (data not shown). The DNA sequence of the ITS-1 and ITS-2 regions contained in the amplified fragment obtained with primers TASF and TASR from two randomly selected isolates (isolates K18 and K19) matched completely

Fig. 4. Agarose gel of PCR products amplified from genomic DNA with the TASF and TASR primers obtained from the 29 clinical *Trichosporon* species isolates. The lane markings correspond to the isolate numbers (see Table 2). Lane M, 100 bp DNA marker, with the positions of 100, 600 and 2000 bp fragments indicated.

(100 %) with the reported sequences of some of the reference strains (CBS 2530, CBS 2479, CBS 7137, CBS 8520 and CBS 8640) of *T. asahii* only and not with other *Trichosporon* species or with other fungi (Table 3, see below). The species-specific identity of the four unidentified isolates was investigated further.

PCR amplification of the ITS regions with the panfungal primers ITS1 and ITS2, ITS3 and ITS4, or ITS1 and ITS4, as well as with the ITS1 and TASR primers, resulted in specific amplification of a single DNA fragment from all four *Trichosporon* isolates, while no amplification was obtained with the TASF and ITS4 primer pair (data not shown). The DNA sequence of the ITS-1 region from all four isolates matched completely (100 %) with the DNA sequence of this region from *T. asteroides* strains CBS 7623 and M 9817 but differed at one nucleotide position from *T. asteroides* strain

CBS 2481 (Table 3). Furthermore, the sequence of the ITS-2 region matched completely (100 %) with the DNA sequence of this region from *T. asteroides* strains CBS 2481, CBS 7623 and M 9817, as well as from two *T. asahii* strains (Table 3).

Fingerprinting of 25 isolates confirmed as *T. asahii* using the GAC-1 RAPD primer yielded eight different patterns, which were arbitrarily referred to as patterns A–H (Fig. 5a). Only isolate K1 and K5 yielded the unique A and H patterns, respectively. Three (K2, K3 and K4), two (K7 and K25), six (K9, K13, K14, K15, K16 and K22), four (K6, K26, K27 and K28), three (K8, K19 and K24) and five (K10, K11, K12, K17 and K18) isolates exhibited the B, C, D, E, F and G patterns, respectively. All four *T. asteroides* isolates exhibited a single pattern using the GAC-1 RAPD primer, which was distinct from the patterns exhibited by the *T. asahii* isolates (Fig. 5a,

Table 3. Specific nucleotides present at various positions in the ITS-1 and ITS-2 regions among reference strains of *T. asahii* and *T. asteroides* and clinical isolates from Kuwait

<i>Trichosporon</i> species	Isolate no.	GenBank accession no.	Nucleotide in ITS-1 at:			Nucleotide in ITS-2 at:
			Position 12	Position 15	Position 56	Position 53
<i>T. asahii</i>	CBS 2479	AB018013	T	A	C	T
	CBS 2482	AF444434	T	A	T	C
	CBS 2530	AB018014	T	A	C	T
	CBS 4828	AF444419	T	A	C	C
	CBS 7137	AF444466	T	A	C	T
	CBS 8520	AF444457	T	A	C	T
	CBS 8640	AF444465	T	A	C	T
	ST8H-6a	AY359870	T	A	C	T
	wb466	AF455425	T	A	C	T
	K18	AJ864866	T	A	C	T
	K19	AJ864867	T	A	C	T
<i>T. asteroides</i>	CBS 2481	AF444416	A	T	T	C
	CBS 7623	AB018018	A	T	C	C
	M 9817	AB031521	A	T	C	C
	K20	AJ864868	A	T	C	C
	K21	AJ864869	A	T	C	C
	K23	AJ864870	A	T	C	C
	K29	AJ864871	A	T	C	C

Fig. 5. Representative agarose gels of fingerprinting patterns from RAPD analyses of genomic DNA with the GAC-1 primer from selected *T. asahii* isolates and all four *T. asteroides* isolates (a) and with the M13 primer from the four *T. asteroides* isolates (b). The lane markings correspond to the isolate numbers (see Table 2). Lane M, 100 bp DNA marker, with the positions of 100, 600 and 2000 bp fragments indicated.

pattern I). However, RAPD analysis performed with the M13 primer exhibited heterogeneity among the four *T. asteroides* isolates (Fig. 5b).

DISCUSSION

The taxonomy of *Trichosporon* is evolving fast (Fell & Scorzetti, 2004; Middelhoven *et al.*, 2004). Several new taxa have recently been proposed for inclusion in the genus (Fell & Scorzetti, 2004; Middelhoven *et al.*, 1999, 2000, 2001; Sugita & Nakase, 1998; Sugita *et al.*, 2002b). *Trichosporon* species are emerging opportunistic pathogens of immunocompromised patients, particularly those with granulocytopenia (Fleming *et al.*, 2002; Walsh *et al.*, 2004). The number of *Trichosporon* species causing disseminated disease is expanding; *T. asteroides* and *T. loubieri* have recently been shown to cause disseminated trichosporonosis (Kustimur *et al.*, 2002; Marty *et al.*, 2003; Padhye *et al.*, 2003). Disseminated trichosporonosis is associated with high mortality, despite antifungal therapy. Since *Trichosporon* species may have different antifungal susceptibilities (McGinnis *et al.*, 1998; Paphitou *et al.*, 2002), it is important to identify them accurately for specific therapy. Identification based solely on carbon and nitrogen assimilation profiles may not be totally reliable (Middelhoven *et al.*, 2004).

In this study, we have presented data on the phenotypic and molecular identification of *Trichosporon* species recovered from various clinical specimens in Kuwait. Although *Trichosporon* isolates were collected over a 6-year period from 29 different patients, only two colony types were recognized, which corresponded to *T. asahii* and *T. asteroides*. This lack of species diversity in our isolates may be attributed to the types of specimen cultured. Most of our isolates originated from urine and blood specimens (79%) and none from superficial sites such as skin or hair, which may have a different species spectrum (Lee *et al.*, 1990). It is interesting

that three of the four isolates exhibiting a different colony type originated from blood and were later identified as *T. asteroides*. They also showed cracking of the agar after 7 days of incubation at 28 °C, a phenomenon seen mostly among *T. inkin* isolates (Gueho *et al.*, 1994); this has not been described for isolates of *T. asteroides*.

Although all of our isolates were identified by the Vitek 2 system as *T. asahii*, their assimilation profiles were not identical. Utilization of D-galactose, D-gluconate, isoleucine arylamidase and β -xylosidase and non-utilization of D-melezitose, D-sorbitol, D-mannitol, L-rhamnose and citrate was shown by the *T. asteroides* isolates, and some of these profiles were also exhibited by a few *T. asahii* isolates. In this context, it should be mentioned that, while phenotypic characteristics including assimilation of a large number of carbon and nitrogen compounds form the basis for species identification of yeasts, the inconsistency of assimilation results has been a problem. The Vitek 2 system is programmed to identify only three species of *Trichosporon*, namely, *T. asahii*, *T. inkin* and *T. mucoides*. Consequently, strains may be misidentified, and genetically distinct species could be overlooked. The application of modern molecular methods including sequencing of rRNA genes offers a reliable alternative to overcome this difficulty (Fell & Scorzetti, 2004; Middelhoven *et al.*, 2004).

Previous studies have shown that *T. asahii* can rapidly be identified by PCR using *T. asahii*-specific primers (Sugita *et al.*, 1998b, c, 2001b). These studies have targeted either the divergent region of the 26S rRNA gene or the more variable ITS region, located between the 18S and 26S rRNA genes and comprising ITS-1 (between the 18S and 5.8S rRNA genes) and ITS-2 (between the 5.8S and 26S rRNA genes) (Fig. 1). Other targets that show species-specific variations among *Trichosporon* species have not been explored, mainly due to the limited number of molecular studies carried out. We have

also established, in this study, a PCR assay for specific detection of DNA from *T. asahii* and *T. mucoides*. Although the *T. asahii*-specific forward primer (TASF) has been used previously, the reverse primers used in earlier studies were either panfungal or homologous to sequences from several *Trichosporon* species (Sugita *et al.*, 1998b, c, 2001b). However, the primers for the detection of *T. mucoides* DNA have not been described previously.

The amplification of DNA from 25 of 29 clinical isolates from Kuwait with *T. asahii*-specific primers suggested that only 25 isolates were actually *T. asahii* strains, while the remaining four isolates remained unidentified. The ITS-1 and ITS-2 regions are highly conserved among *Trichosporon* species and sequencing of these two regions is sufficient to establish the identity of *Trichosporon* or other fungal organisms (Sugita *et al.*, 1999, 2002a). DNA sequencing of the ITS-1 and ITS-2 regions from two of the 25 isolates confirmed the species-specific identity of these isolates. Direct DNA sequencing of the ITS-1 and ITS-2 regions obtained with panfungal primers also confirmed the identity of the four discrepant isolates as *T. asteroides*. The ITS-1 and ITS-2 regions from *T. asahii* and *T. asteroides* are highly conserved. The reported sequence of the ITS-1 region from *T. asahii* differs from the *T. asteroides* sequence in two or three nucleotide positions (one nucleotide is the same in some reference strains of *T. asahii* and *T. asteroides*), while the sequence of the ITS-2 region is either identical or varied at a single nucleotide position from these two *Trichosporon* species (Sugita *et al.*, 1999, 2002a) (Table 3). The TASF primer is *T. asahii*-specific as the two nucleotides at the 3' end are different in *T. asteroides*, while the sequence of TASR is identical in *T. asahii* and *T. asteroides* (Sugita *et al.*, 1999). On the basis of the above observations, *T. asahii* and *T. asteroides* appear to be closely related species (Middelhoven *et al.*, 2004).

The fingerprinting analyses using RAPD primers, shown previously to yield variable patterns for clinical *T. asahii* isolates (Sugita *et al.*, 2001a), demonstrated genetic diversity among the *Trichosporon* isolates from Kuwait. Interestingly all four *T. asteroides* isolates clustered together and exhibited a unique pattern with the GAC-1 primer. However, some heterogeneity among these strains was revealed using the M13 primer. Only one previous study carried out in Japan has investigated genetic diversity among clinical and environmental isolates of *Trichosporon* species (Sugita *et al.*, 2001a). The data obtained with three RAPD primers showed limited diversity among clinical *Trichosporon* species isolates, as the similarity of polymorphic bands was very high (91 %).

Considering the fact that Kuwait has a heterogeneous population representing different nationalities, it was anticipated that a wide spectrum of medically important species might occur in clinical specimens. However, this study reinforces the current view that *T. asahii* is the most common species associated with human clinical specimens and has a wide geographic distribution. Moreover, the currently available Vitek 2 yeast identification system is not adequately updated to identify correctly all the clinically relevant

Trichosporon species, and direct DNA sequencing of the rRNA gene ITS region is required to achieve definitive identification.

ACKNOWLEDGEMENTS

This study was supported by Research Administration grants YM02/02, MI 118 and the College of Graduate Studies, Kuwait University. We thank Zaiba Khan for technical assistance.

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