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Genotyping of *Trichosporon asahii* strains isolated from urinary tract infections in a Turkish university hospital

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Aim: *Trichosporon* infections are associated with a wide spectrum of clinical manifestations. *Trichosporon asahii* is the most important species in the genus. Our aim was to evaluate the genotypic relatedness of urinary *T. asahii* strains isolated from immunocompetent patients during a hospital outbreak.

Materials and methods: Twenty-three *T. asahii* isolates obtained from 15 patients hospitalized in different wards of Ankara University Ibn-i Sina Hospital between July 2004 and April 2005 were studied. *T. asahii* was identified by API 20C AUX (BioMérieux Vitek, Hazelwood, USA) system and confirmed by genus and species specific polymerase chain reactions. Genetic relatedness of the isolates was evaluated by randomly amplified polymorphic DNA (RAPD) and repetitive element PCR (REP-PCR) analyses.

Results: RAPD analysis divided the isolates into 5 groups. The results of REP-PCR correlated well with the data obtained by RAPD analysis, although this method produced 3 different groups.

Conclusion: In this study of urinary trichosporonosis, 19 of the 23 isolates were found to be genotypically related, suggesting that a single genotype was prevalent in our hospital environment. The genotypically unrelated 4 isolates were probably of endogenous origin. RAPD and REP-PCR analyses can be used for genotyping *T. asahii* strains.

Key words: *Trichosporon asahii*, genotyping

Bir üniversite hastanesinde idrar yolu enfeksiyonlarından izole edilen *Trichosporon asahii* suşlarının genotiplendirilmesi

Amaç: *Trichosporon* infeksiyonları çok çeşitli klinik görünümler ile birlikte seyretmektedir. *Trichosporon asahii* bu cins içerisindeki en önemli türdür. Bu çalışmada, bir hastane salgını esnasında bağışıklık sistemi baskılanmamış hastalardan izole edilen üriner *T. asahii* suşlarının genotipik yakınlıklarının araştırılması amaçlanmıştır.

Yöntem ve gereç: Temmuz 2004 – Nisan 2005 tarihleri arasında Ankara Üniversitesi İbn-i Sina Hastanesi'nin farklı kliniklerinde yatan 15 hastadan izole edilen 23 *T. asahii* izolatı ile çalışılmıştır. *T. asahii* identifikasyonu API 20C AUX (BioMérieux Vitek, Hazelwood, ABD) ile yapılmış, cins ve tür spesifik polimeraz zincir reaksiyonu ile doğrulanmıştır. İzolatların genetik yakınlıkları randomly amplified polymorphic DNA (RAPD) ve repetitive element PCR (REP-PCR) yöntemleri ile değerlendirilmiştir.

Bulgular: RAPD analizi izolatları 5 farklı gruba ayırmıştır. REP-PCR sonuçları ile 3 farklı grup elde edilmekle birlikte iki yöntemin verileri birbiri ile uyumlu bulunmuştur.

Sonuç: Bu Türkiye'deki üriner trikosporonozis çalışmasında, 23 izolatın 19'unun genotipik olarak birbiri ile uyumlu bulunması, hastane ortamında tek bir genotipin baskın olarak bulunduğunu göstermektedir. Genotipik olarak birbiriyle uyumsuz bulunan diğer 4 izolatın endojen floradan kaynaklandığı düşünülmüştür.

Anahtar sözcükler: *Trichosporon asahii*, genotiplendirim

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Introduction

Trichosporon is a medically important genus with a widespread natural distribution, being found in soil and water and on plants. It is also carried on the mucosal and cutaneous surfaces of humans (1). Today, 38 species are included in this genus, several of which are considered human pathogens: *T. asahii* (previously known as *Trichosporon beigelii*), *T. asteroids, T. ovoides*, *T. inkin*, *T. cutaneum*, *T. mucoides*, *T. japonicum*, and *T. louberi* (2). Among them, *T. asahii* and *T. mucoides* are associated with deep seated infections, while *T. inkin* and *T. ovoides* are associated with piedra of the head and genital areas, respectively (3).

The first report on disseminated trichosporonosis was published in 1970 as the causative agent of a brain abscess in a leukemic patient (4). Since then, sporadic cases have been reported over the years, especially in patients with hematological malignancies (5-8). Less commonly reported risk factors associated with infections caused by this agent include treatment with immunosuppressive drugs, transplantation, AIDS, extensive burns, and the presence of implanted prosthetic and artificial medical devices. Disseminated *Trichosporon* infections in immunocompromised patients are frequently fatal, despite therapy with amphotericin B (7,9-11). The microorganism can also be found in asymptomatic patients, in this case representing a colonization that must be kept under control as well, in particular in neutropenic patients (1).

In immunocompetent healthy individuals, *Trichosporon* species cause superficial infections of the skin and mucous membranes. Non-immunosuppressed patients have also been reported to suffer from *Trichosporon* infections (1,7,9,10). In some of these patients, *Trichosporon* strains were associated with infections due to ophthalmologic surgery, existence of prosthetic devices, intravenous drug abuse, peritoneal dialysis, diabetes mellitus, and intensive-care stay (1,9,12). There are also cases with no identifiable underlying disease (7,13). In such cases, the infection may begin as a local colonization of fungus, spread on mucous membranes and possibly lead to systemic infection (6,7,9,10). Most cases of trichosporonosis appear to be derived from endogenous reservoirs of the organism in the gastrointestinal tract (14). Factors that enhance mucosal colonization and subsequent invasion of *Trichosporon* species include broad-spectrum antibiotic treatment and breaks in mucosal barriers (9).

Although the finding of *Trichosporon* yeasts in the hospital environment (air, water, surfaces etc.) is not frequent, some reports exist regarding the isolation of yeast from endoscopic forceps and from the air in the endoscopy room (13), from a cocaine solution used for local anesthesia during bronchoscopy (15), and from the distal portion of the urine catheter collection system (16).

In recent years, case reports on trichosporonosis from Turkey have also been published. These reports describe patients with underlying factors such as bone marrow transplantation (17), acute leukemia (18), non-hematological malignancy (19), and prematurity (20).

Genotyping of an infectious agent is usually performed for assessing the genetic relatedness of the strains, especially for epidemiological purposes. Although there are many molecular methods for genotyping *Candida* and *Cryptococcus* species, only a few methods are used for typing *Trichosporon* spp. These methods include random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), gene sequencing, and glucuronoxylomannan polysaccharide antigen analysis (2,9,13,21,22).

In the present study, we report on the recovery of *T. asahii* from urine samples of non-granulocytopenic patients and evaluation of the genotypic relatedness of the strains by 2 different polymerase chain reaction (PCR)-based molecular techniques, i.e. RAPD analysis with M13 primer and repetitive-element PCR (REP-PCR) analysis with ERIC2 primer.

Materials and methods

A total of 25 *T. asahii* urinary strains stored in Microbank (Pro-Lab Diagnostics, UK) at –20 °C after initial isolation were studied retrospectively. These isolates were obtained from urinary catheters of 17 immunocompetent patients (11 male, 6 female, median age 44) hospitalized at Ankara University Ibn-

i Sina Hospital between July 2004 and April 2005. None of the patients were neutropenic during the period when *T. asahii* was isolated, but all of them had urinary catheters. Among these patients, the isolates of 23 were subjected to genotyping, as their isolates were identified as *T. asahii* by molecular techniques. These patients were hospitalized in the neurosurgery, urology, nephrology, orthopedics, and reanimation clinics. Patient characteristics and underlying diseases are shown in Table 1.

Trichosporon spp. was suspected when yeast forms were seen in the urinary precipitate and budding yeast cells and arthroconidia were observed in the cultures. The initial *T. asahii* identification was made using the API 20C AUX (BioMérieux Vitek, Hazelwood, USA) system and all strains were subjected to PCR using genus and species specific primers as previously described for confirmation (7,23).

Antifungal susceptibilities of the isolates were calculated by minimal inhibitory concentration (MIC) determination for fluconazole (Flu), itrakonazole (Itra), and amphotericin-B (ampB) by E-test (AB Biodisk, Solna, Sweden). The MIC values were defined as the lowest drug concentration that resulted in complete inhibition of visible growth (9,24).

Genotyping of *T. asahii* strains was performed by RAPD and REP-PCR methods using the M13 (5'-GAG GGT GGC GGT TCT-3') and ERIC2 (5'-AAG TAA GTG ACT GGG GTG AGC G-3') primers, respectively. RAPD analysis was performed at 40 °C annealing temperature as described in the literature (8,9,13,21). REP-PCR analysis was performed using only the ERIC2 primer as previously mentioned in the literature with minor modifications to the master mix preparation and cycling conditions (25,26). Briefly, the reaction was performed in a 25 µL total volume containing 25 pmol of the primer, 2.5 µL of 10× PCR buffer (10 mM tris, 50 mM KCl; pH 8.3), 200 µM dNTP each, 2.5 mM $MgCl_2$, 1 U of *Taq* DNA polymerase (Fermantas, Vilnius, Lithuania), and 30 ng of template DNA. Cycling conditions consisted of an initial denaturation step at 95 °C for 3 min, followed by 30 cycles of denaturation at 90 °C for 30 s, annealing at 52 °C for 1 min, and extension at 72 °C for 1 min. A final extension was performed at 72 °C for 5 min.

The PCR products of the isolates were run on 1.5% agarose gel electrophoresis at 100 V for approximately 2 h. The resulting band patterns were visualized under UV after ethidium bromide staining of the gels.

Cluster analysis of RAPD and REP-PCR patterns were obtained by Gene Directory gel analysis software (Syngene, Cambridge, UK) with the unweighted pair group method using Dice's coefficient (Figures 1 and 2, respectively).

Results

All of the 25 *Trichosporon* isolates that were identified as *T. asahii* by the API system gave amplicons using *Trichosporon* genus specific primers. When species specific PCR was performed, 23 of the isolates obtained from 15 patients gave amplicons. MIC values of these 23 isolates are given in Table 2. As most of the isolates were clustered in a single genotype with each method used, and the rest of the groups consisted of only one isolate, we did not evaluate the relationship between the genotypes and antifungal susceptibilities.

The 23 isolates that were identified as *T. asahii* by species specific PCR were subjected to genotyping. RAPD analysis by M13 primer resulted in 7 groups showing different band patterns. Three of these groups were found to share approximately 95% similarity by cluster analysis; thus they were considered a single genotype. As a result, RAPD analysis yielded 5 genotypes: 4 of the genotypes were composed of a single isolate and the fifth genotype contained 19 isolates (Table 1).

REP-PCR analysis produced 4 groups producing different band patterns. Two of these groups shared a 95% similarity by cluster analysis, and were regarded as belonging to the same genotype. As a result, REP-PCR analysis produced 3 genotypes: 2 of the genotypes contained single isolates that corresponded to the 2 different genotypes of RAPD analysis. The third genotype consisted of 21 isolates containing the 2 isolates that were distributed to different genotypes by RAPD analysis (Table 1).

Table 1. Characteristics of the patients from whom *T. asahii* was isolated as the causative agent of urinary tract infection and genotypes of the isolates.

Patient No.	Age, sex	Wards	Underlying Disease	WBC Count (per mL) (neutrophil %)	Sample No.	Isolation Date	RAPD Genotype (Band pattern no*)	REP-PCR Genotype (Band pattern no.**)
1	25, M	Neurosurgery	Infiltrative glioma - operated	21,900 (92.7)	189	11.07.2004	**I (1)**	**I (2)**
2	40, M	Neurosurgery	Subarachnoidal hemorrhage - operated	28,000 (80.4) 16,900 (79.3)	190 196	12.07.2004 17.07.2004	**I (1)** **I (1)**	**I (1)** **I (2)**
3	67, F	Neurosurgery	Intracranial tumor - operated	21,000 (90.9)	229	17.08.2004	**I (3)**	**I (2)**
4	58, M	Neurosurgery	Intracranial aneurism - operated	12,310 (86.4) 15,320 (87.5) 17,720 (89.7)	236 237 243	29.08.2004 30.08.2004 05.09.2004	**I (1)** **I (1)** **V (7)**	**I (2)** **I (2)** **III (4)**
5	44, F	Neurosurgery	Intracranial tumor - operated	19,900 (80.2)	223	14.08.2004	**III (5)**	**I (1)**
6	55, M	Urology	Left ureter tumor - operated	11,110 (78.7)	259	27.09.2004	**IV (6)**	**II (3)**
7	42, M	Reanimation	Hypoxic encephalopathy - bee sting	11,600 (78) 7900 (58.3)	270 376	06.10.2004 23.01.2005	**I (1)** **I (2)**	**I (2)** **I (1)**
8	62, M	Reanimation	Traumatic subaracchnoidal hemorrhage	9400 (75.0)	302	01.11.2004	**I (1)**	**I (2)**
9	45, M	Reanimation	Hypoxic encephalopathy	14,550 (88.2) 15,630 (89.3)	310 318	14.11.2004 21.11.2004	**I (1)** **I (1)**	**I (1)** **I (2)**
10	43, F	Orthopedics	Femur fracture - operated	18,320 (85.4) 17,240 (83.2)	349 374	03.01.2005 19.01.2005	**II (4)** **I (1)**	**I (1)** **I (2)**
11	20, M	Reanimation	Traffic accident - multiple injury	17,240 (81.9)	375	24.01.2005	**I (3)**	**I (2)**
12	24, F	Neurosurgery	Pituitary adenoma - operated	17,300 (86) 16,230 (85.8)	432 435	28.02.2005 02.03.2005	**I (1)** **I (1)**	**I (2)** **I (2)**
13	39, F	Reanimation	Suicide attempt - multiple injury	18,430 (86.4) 19,550 (87.5)	449 453	18.03.2005 23.03.2005	**I (1)** **I (1)**	**I (2)** **I (1)**
14	45, M	Reanimation	Traffic accident - multiple injury	19,600 (76.8)	450	20.03.2005	**I (1)**	**I (2)**
15	51, M	Neurosurgery	Subaracchnoidal hemorrhage - operated	7000 (76.7)	470	21.04.2005	**I (1)**	**I (1)**

* Isolates with RAPD band patterns of 1, 2, and 3 were found to share 94%-95% similarity when cluster analysis was performed, therefore grouped in the same genotype.
** Isolates with REP-PCR band patterns of 1 and 2 were found to share ~95% similarity when cluster analysis was performed, therefore were grouped in the same genotype

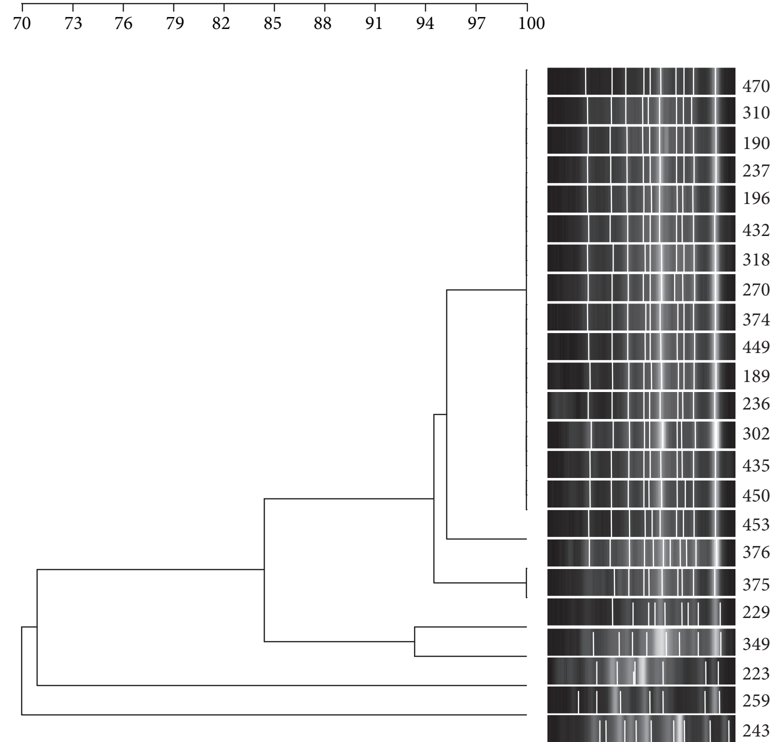

Figure 1. Dendrogram of RAPD band patterns generated with primer M 13 from *T. asahii* strains.

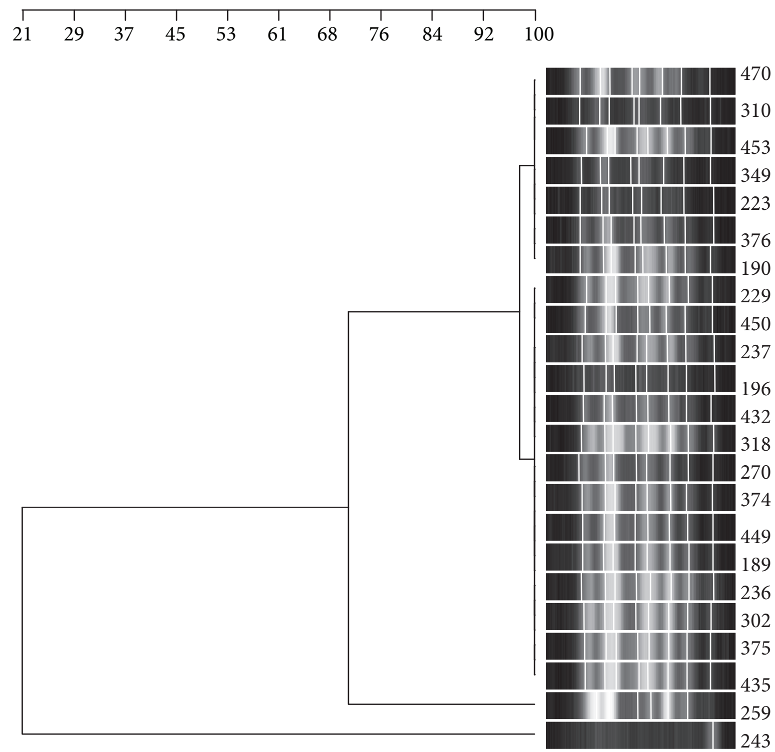

Figure 2. Dendrogram of REP-PCR band patterns generated with primer ERIC 2 from *T. asahii* strains.

Table 2. E-test results of the study strains.

Sample No.	Results of E-test (mg/L)		
	Flu	**Itra**	**AmpB**
189	4	0.5	0.125
190	2	0.25	0.25
196	4	1	0.5
229	4	0.5	1
236	16	0.125	0.125
237	4	0.25	0.25
243	8	1	0.125
223	4	0.5	0.5
259	2	0.125	0.06
270	4	1	1
376	8	1	0.25
302	8	1	1
310	16	0.5	0.25
318	8	0.03	0.125
349	4	0.25	0.25
374	8	0.5	0.06
375	4	1	2
432	8	0.25	0.25
435	4	0.5	0.5
449	32	0.5	1
453	8	0.5	0.5
450	64	2	1
470	2	0.25	1
C. albicans			
ATCC 26555	2	0.125	0.06
Defined E-test MIC ranges in previous studies(9,24)	*0.25-128*	*0.015-4*	*0.015->32*
Defined microbroth dilution MIC ranges in previous studies (2,9,12,24)	*0.125-64*	*0.015-4*	*0.125->32*
Susceptibility breakpoints (9)	***2-8***	***0.125***	***1***

Discussion

Trichosporon asahii has become an important etiological agent of systemic disease, especially in immunocompromised patients. Reports on its isolation in previously immunocompetent patients have been increasing in recent years. Correct characterization of *Trichosporon* species can be significant at the therapeutic level in view of their distinct antifungal susceptibility profiles, particularly those of *T. asahii*, which is highly resistant to amphotericin B in-vitro. Early diagnosis and treatment are therefore of paramount importance in trichosporonosis patients (12,23). Although the identification of the *Trichosporon* is generally made by physiological and morphological techniques, they are time-consuming and usually inadequate to identify *Trichosporon* spp. (2,3,12). Commercial kits based on the assimilation of carbon and nitrogen compounds for identification may result in misidentification of the isolates, particularly at the species level (2). By using the API 20 C AUX system, *T. asahii* and *T. inkin* were identified. However, for the other species, it is of little value and has to be supplemented with other phenotypic and molecular methods. Molecular investigations, such as PCR with species-specific primers, have recently been developed with the aim of applying a more simple, specific and faster technology for mycological diagnosis (3,22). The main advantages of molecular methods are their high sensitivity and specificity, and their being fully discriminative even for closely related species (12).

In our study 2 (8%) of the 25 *Trichosporon* isolates that were initially identified as *T. asahii* by the API 20 c AUX system did not yield any amplicons by *T. asahii* species specific primers. In the study by Rodriguez-Tudela et al. (12), 7 (46.6%) of the 15 biochemically identified *T. asahii* strains were found to belong to different species (namely, *T. faecale, T. coremiiforme, T. cutaneum*, and *T. japonicum*) by molecular methods. Our results also indicate that molecular techniques are more informative than conventional identification methods for species identification of *T. asahii*.

In non-neutropenic patients, trichosporonosis begins as a local colonization of fungus (generally derived from an endogenous reservoir of organism in the gastrointestinal tract), spreads on mucous membranes, and possibly leads to systemic infection (6,7,9,10,14). Factors that enhance mucosal colonization and subsequent invasion of *Trichosporon* species include broad-spectrum antibiotic treatment and breaks in mucosal barriers (9). In our study, none of the patients were neutropenic during the period when *T. asahii* was isolated, but all of them had urinary catheters and most were receiving antibiotic treatment, which can be regarded as risk factors for developing urinary *T. asahii* infection.

Thirteen (86%) of the patients in our study group were hospitalized in the neurosurgery or reanimation clinics during the study period. The first *T. asahii* isolates of our study were cultured from 5 patients hospitalized in the neurosurgery clinics in July and September 2004. After October 2004, most of the isolates were obtained from the patients hospitalized in the reanimation clinic, and almost all of these isolates shared the same genotype with the neurosurgery isolates. There is a high rate of patient and consulting staff transfer between these 2 clinics, which may explain the clustering of the patients in these clinics. Based on the data obtained from the patients' files, only 1 patient (patient 15) was transferred between these 2 clinics during our study period. This patient was operated on by the neurosurgery staff on 24th March, and transferred to the reanimation clinic on 29th March. On 13th April, he was again transferred to the neurosurgery clinics. On the other hand, we do not know the consultation data of the staff between these 2 clinics, except for patient 12. This neurosurgery patient was consulted and intubated by the staff of the reanimation clinic 3 days before the isolation of *T. asahii* from her urine culture. All of the patients in these 2 clinics had urinary catheters, and all of them were receiving antibiotic treatment due to wound/urine/respiratory system bacterial infections or bacterial sepsis (most of them due to coagulase negative staphylococci, or gram negative rods). Patients 6 and 10 were hospitalized out of these 2 clinics (in the urology and orthopedics clinics, respectively) during the study period. Only patient 6 had complaints of urinary tract infection (fever, lower abdominal pain, and turbidity of the urine). His blood culture was sterile, but there was also a 10^5 CFU/mL bacterial growth (*E. coli*) in his urine culture. The isolate of patient 6 was found to belong to a different genotype by both genotyping methods, while the genotypes of the isolates of patient 10 were similar with the common genotype of the other isolates.

Our genotyping results indicate that at least 19 (82.6%) of the 23 *T. asahii* strains were of the same genotype. We assume that one genotype of *T. asahii* was prevalent in our hospital setting during this outbreak, but different genotypes also existed in clinics other than the neurosurgery and reanimation clinics, that originated either from this prevalent genotype (orthopedics patient) or from the endogenous flora of the patients (urology patient). As we performed this study retrospectively on the isolates of the patients, we cannot make any speculations about the origin of the strains, as no samples were obtained from the staff, air, or inanimate objects in the clinics of these patients. There seems to be a hospital outbreak beginning in June 2004 and lasting until April 2005, involving the neurosurgery and reanimation wards. The causative agent might have been transferred between the neurosurgery and reanimation clinics, most probably by the staff.

When the results of RAPD and REP-PCR analyses were compared, it was observed that RAPD analysis resulted in 5 different genotypes, while REP-PCR analysis yielded 3 genotypes. Nineteen isolates were found to cluster in the same genotype by RAPD analysis, while 4 others were of different genotypes. Isolate no. 243 (genotype V) was the third isolate of patient 4, whose previous isolates belonged to the

common genotype (genotype I). This isolate was cultured 1 week after his second isolate. This isolate was also found to be genotypically different by REP-PCR analysis from the previous isolates of the same patient (genotype III and genotype I, respectively).

Isolate no. 349 was the initial isolate of patient 10. RAPD analysis clustered this isolate in another genotype (genotype II) with the next isolate (no. 374, genotype I) cultured 2 weeks after the first isolate. REP-PCR, on the other hand, grouped these 2 isolates in a single genotype, displaying a 95% similarity. This isolate may have arisen from its ancestral isolate with minor changes in its genome, which may have been overlooked by REP-PCR.

Our results indicate that RAPD analysis may be more sensitive in determining minor changes in the *T. asahii* genome. On the other hand, genotypically unrelated isolates of RAPD analysis may have arisen from the prevalent genotype of the hospital environment by minor modifications, some of which could not be differentiated by the REP-PCR analysis.

As a result, we conclude that molecular techniques are useful in identifying *T. asahii* isolates at species level. Although genotyping *Trichosporon* strains by sequencing the intergenic spacer (IGS) regions is the most discriminative method today (2), not all clinical laboratories have sequencing options for routine analysis. Genotyping of strains can be done by RAPD or REP-PCR analysis in laboratories using PCR techniques. These methods can give informative data about the relatedness of the strains, especially if they are performed during an outbreak.

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