



Strain Typing of *Trichosporon asahii* Clinical Isolates by Random Amplification of Polymorphic DNA (RAPD) Analysis

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Abstract

Objective The aim of this study was to identify and isolate *Trichosporon asahii* (*T. asahii*) from clinical samples and to assess the genetic relatedness of the most frequently isolated strains of *T. asahii* using random amplification of polymorphic DNA (RAPD) primers GAC-1 and M13.

Methods All the clinical samples that grew *Trichosporon* species, identified and confirmed by polymerase chain reaction (PCR) using *Trichosporon* genus-specific primers, were considered for the study. Confirmation of the species *T. asahii* was carried out by *T. asahii*-specific PCR. Fingerprinting of the most frequently isolated *T. asahii* isolates was carried out by RAPD using random primers GAC-1 and M13.

Results Among the 72 clinical isolates of *Trichosporon* sp. confirmed by *Trichosporon*-specific PCR, 65 were found to be *T. asahii* as identified by *T. asahii*-specific PCR. Fingerprinting of the 65 isolates confirmed as *T. asahii* using GAC-1 RAPD primer yielded 11 different patterns, whereas that of M13 primer produced only 5 patterns. The pattern I was found to be the most predominant type (29.2%) followed by pattern III (16.9%) by GAC-1 primer.

Conclusions This study being the first of its kind in India on strain typing of *T. asahii* isolates by adopting RAPD analysis throws light on genetic diversity among the *T. asahii* isolates from clinical samples. Fingerprinting by RAPD primer GAC-1 identified more heterogeneity among the *T. asahii* isolates than M13.

Keywords

- strain typing
- *Trichosporon asahii*
- genetic diversity
- RAPD

Introduction

Trichosporon spp. is emerging yeast that is extensively distributed in nature. They form a part of the normal human flora of the mucosal and cutaneous surfaces of humans.¹ The infections caused by *Trichosporon* spp. in humans can be superficial, mucosa-associated, or deep-seated infections,

and are collectively described as “Trichosporonosis.”² In immunocompetent healthy individuals, *Trichosporon* spp. may cause superficial infections like white piedra or skin infections. However, in severely immunocompromised patients such as hematological malignancy patients and solid organ transplant recipients, *Trichosporon* spp. can cause invasive infections.^{3,4} *Trichosporonosis* is frequently

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associated with resistance to 5-fluorocytosine, fluconazole, and amphotericin B. Also, echinocandins have little or no activity against *Trichosporon* spp. Hence, these infections are usually difficult to treat and are associated with high morbidity and mortality rate.⁵ The therapies are only effective if the disease is detected at an initial stage, and, therefore, early diagnosis plays an important role in the successful management of patients with disseminated trichosporonosis. Unfortunately, difficulties in the identification of *Trichosporon* spp. may lead to delay in starting the patient on correct treatment.^{6,7} DNA-based methods have been extensively used for the accurate identification of *Trichosporon* spp.⁸ In this regard, ribosomal genes represent particularly consistent evaluative markers and include alternating conserved regions (D1/D2 region of the 28S rDNA) and variable regions (ITS and IGS1 regions) that may be useful for species identification and phylogenetic studies.⁹⁻¹¹ In 1998, Sugita et al designed primers that would specifically amplify only *T. asahii* based on the internal transcribed spacer regions of this organism's genome. Amplification products were selectively obtained from only *T. asahii* DNA; the DNAs of other *Trichosporon* species as well as those of other medically relevant yeasts were not amplified.^{10,12}

There are about 51 species of *Trichosporon* that have been identified worldwide so far, among which 16 species are of clinical relevance.¹³ Currently, *T. asahii* is the most frequent reported pathogenic yeast in the genus *Trichosporon*, from patients with superficial and invasive trichosporonosis.¹⁴⁻¹⁷

Species identification and molecular typing have become critical elements of nosocomial fungal outbreak investigations. These studies reveal the possible source of nosocomial infection, genetic comparison between invasive or noninvasive isolates, and comparison of the genotypes.¹⁸ 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), restriction fragment length polymorphism, amplified fragment length polymorphism, gene sequencing, and glucuronoxylomannan polysaccharide antigen analysis.¹⁹⁻²⁴

To the best of our knowledge, no data on strain typing and genetic diversity of *Trichosporon* sp. have been reported in India. Hence, in the current study, we report on the isolation of *T. asahii* from various clinical samples and evaluation of the genetic relatedness of these strains by RAPD analysis with GAC-1 and M13 primers.

Materials and Methods

Study design: Hospital-based descriptive study.

Sample collection and study period: Isolates recovered from clinical samples from medical centers across India, over a period of 5 years (July 2011–June 2016), were considered for the study. Out of the total 72 *Trichosporon* isolates that

were processed in our study, 55 were from South India and the remaining were 17 from North India.

Sample source: Out of the 72 isolates from clinical samples, 43 isolates (59.7%) were from urine. Twelve (16.7%) isolates were from blood. Seven isolates (9.7%) grew from samples collected immediately after insertion of percutaneous nephrostomy (PCN) tube. Out of the five (6.9%) respiratory isolates, three were from sputum and two from bronchoalveolar lavage. Four (5.6%) of the *Trichosporon* sp. were isolated from pus, and one isolate (1.4%) grew from peritoneal dialysis (PD) fluid collected from the dialysis bag of a patient who underwent PD.

Phenotypic Characterization

The isolates, which produced dry yeast like colonies on Sabouraud dextrose agar plate, were further characterized by performing gram stain, Dalmau technique, and urea hydrolysis. Urease-positive isolates that showed the presence of blastoconidia, pseudohyphae, and arthroconidia were provisionally identified as belonging to the genus *Trichosporon*.

Preservation of Culture

The cultures were preserved at -20°C on skimmed milk medium until use.²⁵

Genotypic Characterization

For identification of *Trichosporon* isolates to species level (i.e., *T. asahii*) and strain typing, three different polymerase chain reactions (PCRs) were performed with three sets of primers.

DNA Extraction

Genomic DNA from clinical isolates, reference strains of *Trichosporon* species, and *Candida* species (*Trichosporon* reference strains were procured from Microbial Type Culture Collection and Gene Bank [MTCC], India—*T. asahii* MTCC 6179, *T. asteroides* MTCC 7632, *T. cutaneum* var. *cutaneum* MTCC 1963, and *T. jirovecii* MTCC 9036; *Candida* reference strains were from American Type Culture Collection [ATCC]—*Candida albicans* ATCC 90028) were isolated by the method of Mirhendi et al and Vijayakumar et al. Briefly, the culture was suspended in 400 μL TES (2-[Tris-(hydroxymethyl) methylamino]-1-ethane sulfonic acid) lysis buffer (10 mM TRIS [tris(hydroxymethyl)aminomethane] [pH=8], 1 mM EDTA [Ethylenediamine tetraacetic acid] [pH=8], 3% SDS [Sodium Dodecyl Sulfate], and 100mM NaCl) in a microcentrifuge tube and boiled for 1 minute. About 400 μL of phenol:chloroform (1:1) mixture was added, vortexed, and centrifuged at 10,000 rpm for 10 minutes. The aqueous layer was transferred to a fresh microcentrifuge tube and an equal volume of chloroform was added, vortexed, and centrifuged at 10,000 rpm for 10 minutes. The aqueous layer was transferred to another fresh microcentrifuge tube. DNA was precipitated by adding equal volume of ice-cold isopropyl alcohol and washed twice with 70% ethanol. The pellet was dissolved in 40 μL sterile nuclease-free water and stored at -20°C until use.^{26,27}

PCR-Based Identification of *Trichosporon* Species

All the 72 clinical isolates and reference strains were initially subjected to *Trichosporon* specific PCR. This confirmed the identity of all the isolates identified as *Trichosporon* phenotypically, following which *T. asahii*-specific PCR was performed for all the clinical isolates, which identified only *T. asahii*.

Trichosporon-Specific PCR

The first set of primers used were *Trichosporon* genus-specific primers (TRF [forward]—5'AGAGGCCTACCATGGTATCA 3' TRR [reverse]—5'TAAGACCAATAGAGCCCTA 3'). They would specifically amplify only *Trichosporon* species, by aligning with the small subunit of ribosomal DNA sequences. This region is not conserved in other medically important yeasts and hence were not amplified.¹⁰ *Trichosporon*-specific PCR was done by adopting the reaction conditions standardized in our laboratory.²⁸

Trichosporon asahii-Specific PCR

The second sets of primers were *T. asahii*-specific primers (TASF [forward]—5'GGATCATTAGTGATTGC CTTTATA3' TASR [reverse]—5'AGCAGCCTTCAACACAATGGAC3') that identified all the *T. asahii*. The species specificity of the primers was confirmed by previous studies through BLAST search.²⁹ The PCR master mix was prepared containing 25 µL of PCR mix (Takara, Japan), 1 µL of forward (TASF) and reverse primer each (TASR) (GeNei, Bangalore), 1 µL of template DNA, and the volume made up to 50 µL with sterile nuclease-free water. The reaction mixtures were amplified in a thermal cycler (Veriti 96 well, Applied Biosystems, United States), with minor modifications of the PCR reactions conditions (►Table 1), described earlier by Sugita et al, in 1998.¹² The amplified product was electrophoresed on 1.5% (wt/vol) agarose gel, stained with ethidium bromide, and visualized with UV light.

Fingerprinting by RAPD Analysis

DNA fingerprinting of *T. asahii* isolates was carried out by RAPD analysis, using random primers, GAC-1 (5'-GACGACGACGAC-3') and M13 (5'-GAGGGTGGCGGTTCT-3') separately.^{22,30} Both these RAPD primers were optimized separately.^{22,31} The PCR master mix was prepared containing 25 µL of PCR mix (Takara, Japan), 1 µL of GAC-1 or M13 primer (GeNei, Bangalore), 3 µL

of template DNA, and the volume made up to 30 µL with sterile nuclease-free water. The PCR reaction conditions are shown in ►Table 1. Amplified products were separated by 1.5% (wt/vol) agarose gel electrophoresis and similarities and differences among DNA fragment banding patterns were visualized and recorded.³¹

Results

Genotypic Characterization

Trichosporon-Specific PCR

PCR was performed initially with *Trichosporon* genus-specific primers to double check for accurate identification of this genus. This pair of primer confirmed that all the 72 phenotypically identified isolates belonged to the genus *Trichosporon*.²⁸

Trichosporon asahii-Specific PCR

PCR amplification of genomic DNA with *T. asahii*-specific primers resulted in specific amplification of a single DNA fragment at approximately 430bp from 65 clinical *Trichosporon* isolates only, while the remaining seven isolates did not yield an amplified product. *T. asahii* MTCC 6179 also produced band at approximately 430bp (positive control), whereas *T. jirovecii* MTCC 9036 did not yield an amplification product of the DNA isolated (negative control) (►Fig. 1).

Fingerprinting by RAPD

RAPD Genotypes by GAC-1 and Sample Source

Fingerprinting of the 65 isolates confirmed as *T. asahii* strains using GAC-1 RAPD primer yielded 11 different patterns, which were arbitrarily referred to as I to XI (►Fig. 2). The pattern I was found to be the most predominant type in 19/65 (29.2%) isolates, followed by pattern III in 11/65 (16.9%) isolates. Pattern X was the third common pattern found in 9/65 (13.8%) of the isolates. The distributions of the other patterns are shown in ►Table 2. It was observed in our study that the pattern I was the predominant pattern among the urinary isolates 13/38 (34.2%) and pattern X was the most predominant pattern among the blood 3/11 (27.3%), PCN 4/7 (57.1%), and PD fluid isolate 1/1 (100%) (►Table 2). The 17 samples from North India produced patterns (pattern IX–5/17, pattern X–9/17, and pattern XI–3/17) that were different from those produced by the isolates from South India (patterns I–VIII).

Table 1 PCR reaction conditions for identification of *Trichosporon asahii* and its RAPD fingerprinting

Reaction	<i>T. asahii</i> -specific		RAPD GAC-1		RAPD M13	
	Temp	Time	Temp	Time	Temp	Time
Initial denaturation	95°C	7 min	95°C	10 min	95°C	10 min
Denaturation	95°C	30 s	95°C	30 s	95°C	30 s
Annealing	56°C	30 s	45°C	30 s	40°C	30 s
Extension	72°C	30 s	72°C	2.5 min	72°C	2.5 min
Final extension	72°C	10 min	72°C	10 min	72°C	10 min

Abbreviations: PCR, polymerase chain reaction; RAPD, random amplification of polymorphic DNA.

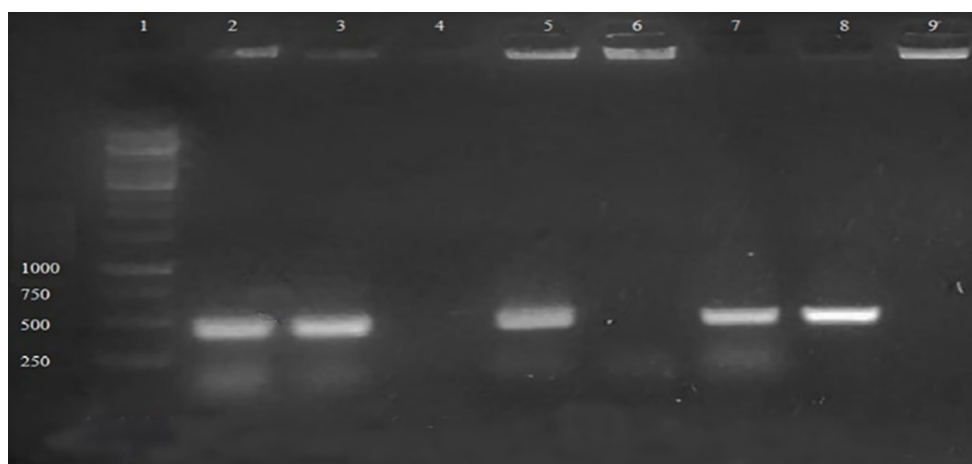


Fig. 1 *Trichosporon asahii*-specific PCR: Lane 1—1kb ladder; Lane 2—*T. asahii* MTCC 6179; Lanes 3, 4, 5, 6, 7, 8—clinical isolates; Lane 9—*T. jirovecii* MTCC 9036

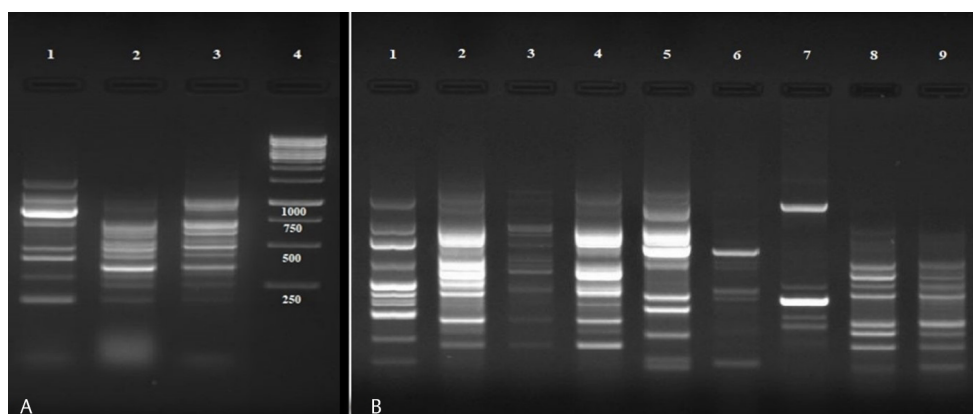


Fig. 2 Fingerprinting by RAPD—GAC-1: Lane A1—pattern IX; Lane A2—pattern XI; Lane A3—pattern X; Lane 4—1 kb ladder. Lane B1—pattern VIII; Lane 2—pattern VII; Lane B3—pattern VI; Lane 4—pattern V; Lane B5—pattern IV; Lane 6—pattern III; Lane B7—pattern II; Lane 8, 9, 10—pattern I.

Table 2 RAPD genotypes by GAC-1 and sample source

Genotypes n = 65	Urine n = 38	PCN n = 7	Blood n = 11	Respiratory n = 5	Pus/wound swab n = 3	PD fluid n = 1
I—19 (29.2%)	13 (34.2%)	0	2 (18.2%)	2 (40%)	2 (66.7%)	0
II—3 (4.6%)	2 (5.3%)	0	1 (9.1%)	0	0	0
III—11 (16.9%)	7 (18.4%)	0	2 (18.2%)	1 (20%)	1 (33.3%)	0
IV—3 (4.6%)	3 (7.9%)	0	0	0	0	0
V—5 (7.7%)	4 (10.5%)	0	1 (9.1%)	0	0	0
VI—2 (3.1%)	2 (5.3%)	0	0	0	0	0
VII—3 (4.6%)	3 (7.9%)	0	0	0	0	0
VIII—2 (3.1%)	2 (5.3%)	0	0	0	0	0
IX—5 (7.7%)	1 (2.6%)	2 (28.6%)	1 (9.1%)	1 (20%)	0	0
X—9 (13.8%)	0	4 (57.1%)	3 (27.3%)	1 (20%)	0	1 (100%)
XI—3 (4.6%)	1 (2.6%)	1 (14.3%)	1 (9.1%)	0	0	0

Abbreviations: PCN, percutaneous nephrostomy; PD, peritoneal dialysis; RAPD, random amplification of polymorphic DNA.

There was also an interesting finding that there was a regional variation in the pattern from North India and South India. The pattern from North was predominantly X, whereas the pattern from South was predominantly I and III.

RAPD Genotypes by M13 and Sample Source

RAPD analysis of the 65 isolates using M13 yielded only five patterns I to V (►Fig. 3). Pattern II was the most common one, that is, 25/65 (38.5%). The next common pattern

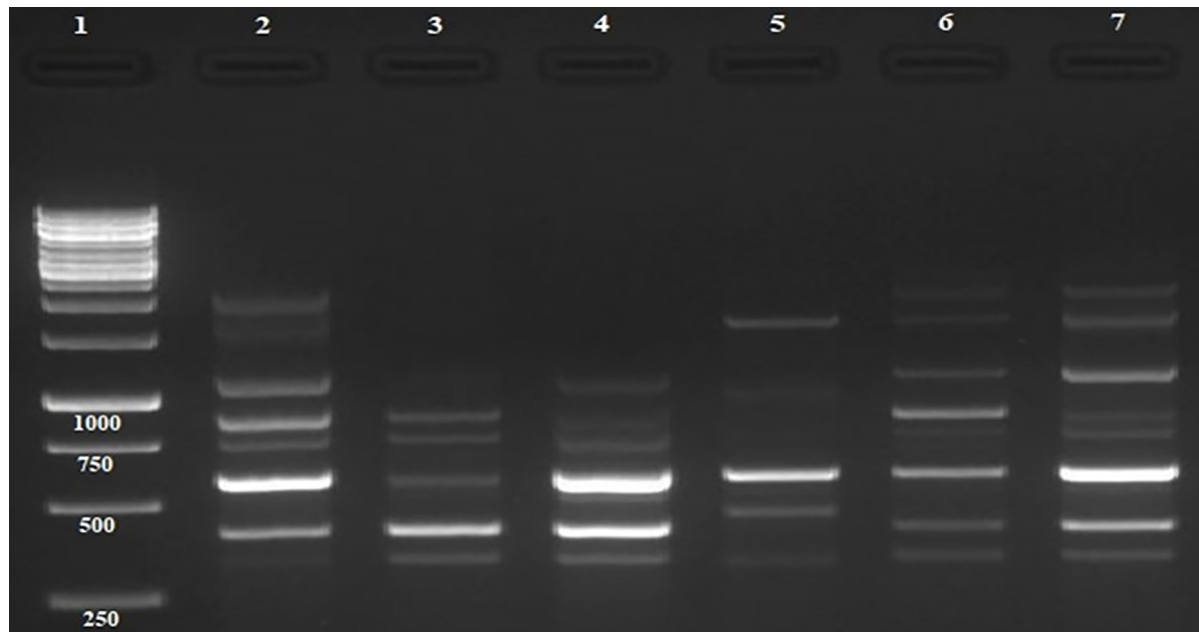


Fig. 3 Fingerprinting by RAPD—M13: Lane 1—kb ladder; Lane 2—pattern V; Lane 3—pattern IV; Lane 4—pattern III; Lane 5—pattern I; Lanes 6, 7—pattern II.

Table 3 RAPD genotypes by M13 and sample source

Genotypes n = 65	Urine n = 38	PCN n = 7	Blood n = 11	Respiratory n = 5	Pus/wound n = 3	PD fluid n = 1
I—15 (23%)	9 (23.7%)	0	4 (36.4%)	1 (20%)	1 (33.3%)	0
II—25 (38.5%)	19 (50%)	1 (14.3%)	1 (9.1%)	2 (40%)	2 (66.7%)	0
III—9 (13.8%)	7 (18.4%)	0	2 (18.2%)	0	0	0
IV—8 (12.3%)	1 (2.6%)	3 (42.9%)	3 (27.3%)	0	0	1 (100%)
V—8 (12.3%)	2 (5.3%)	3 (42.9%)	1 (9.1%)	2 (40%)	0	0

Abbreviations: PCN, percutaneous nephrostomy; PD, peritoneal dialysis; RAPD, random amplification of polymorphic DNA.

was pattern I in 15/65 (23%) of the isolates. Pattern II was common among urine 19/38 (50%) and pus/wound swab isolates 2/3 (66.7%), and pattern I was common among blood isolates 4/11 (36.4%). Among PCN isolates, the most common were pattern IV and pattern V. The distribution of other patterns among the isolates is shown in ►Table 3. The isolates from North India produced predominantly different patterns (pattern IV—8/17, pattern V—8/17) compared to those produced from the isolates from South India (patterns I, II, and III), whereas one PCN isolate from North India produced pattern II that was commonly seen in isolates from South India.

Discussion

Trichosporon sp. being the third most common cause of disseminated yeast infections in humans is usually difficult to diagnose, does not respond to treatment with routinely used antifungal agents, and is associated with high mortality rates.³² Among the *Trichosporon* sp., *T. asahii* is the most common etiological agent causing trichosporonosis, in immunocompromised as well as immunocompetent patients. Early diagnosis and treatment are therefore of utmost importance in trichosporonosis patients.³³

The identification of this genus by phenotypic methods is usually time-consuming and inadequate to identify *Trichosporon* spp.^{34,35} Also, commercially available automated methods based on the assimilation of carbon and nitrogen compounds for identification may result in misidentification of the isolates and hence have to be supplemented with molecular methods. Molecular investigations such as PCR with species-specific primers have been developed with the purpose of applying a more simple, specific, and rapid method for mycological diagnosis of *Trichosporon* sp.^{23,34} The main advantages of these techniques are their high sensitivity and specificity, and at the same time being fully discriminative even for closely related species.

In our study, all the 72 isolates were confirmed as belonging to genus *Trichosporon* using *Trichosporon*-specific PCR, which is a rapid PCR-based molecular method. The primers TRF and TRR were chosen to align with the genus *Trichosporon*. Sugita et al had earlier sequenced the ITS region and described this approach for detection of all species of the emerging yeast, *Trichosporon*.¹⁰ The data from the previous studies also indicate that the PCR detection system is useful for identifying these yeasts.¹² Among these 72 *Trichosporon* isolates, 65 were identified as *T. asahii* by *Trichosporon*

asahii-specific PCR using TASF and TASR primers. The TASF primer is specific for *T. asahii* and has been described previously by Sugita et al,^{11,12} while the sequence of our TASR primer that matched the sequence from five *Trichosporon* species (*T. asahii*, *Trichosporon asteroides*, *Trichosporon japonicum*, *Trichosporon ovoides*, and *Trichosporon aquatile*) was used by Ahmad et al.³⁰ This species-specific PCR helps in the rapid and accurate identification of *T. asahii*, which is the most frequently encountered pathogen among the *Trichosporon* sp.

Overall, *T. asahii* is the most common isolate 65 (90.3%), compared to non-*T. asahii* 7 (9.7%) in our study. This was consistent with the findings of few other studies where *T. asahii* was the predominant isolate.^{36,37}

The fingerprinting analyses of the 65 *T. asahii* strains using RAPD primer GAC-1, which produced 11 different patterns, demonstrated genetic diversity among the *Trichosporon* isolates in our study. However, there was limited diversity among *T. asahii* isolates using RAPD primer M13 that produced only five different patterns. A previous study by Ahmad et al, also demonstrated more genetic diversity among the 25 *T. asahii* isolates by RAPD primer GAC-1, which yielded eight different patterns when compared to M13.³⁰ The results of our study and the previous study indicate that RAPD analysis by GAC-1 may be more sensitive in determining minor changes in *T. asahii* genome. Genetically, diverse isolates of RAPD analysis may have arisen from prevalent genotype of the hospital environment by minor modifications, some of which could not be differentiated by M13 primer.

In our study, out of the 11 different patterns produced by RAPD typing using GAC-1 primer, pattern I (29.2%) was the predominant one followed by pattern III (16.9%). Patterns I and III were the predominant types from strains in South India and pattern X was the predominant one in the strains obtained from North India. Sugita et al were the first to describe five *T. asahii* genotypes, based on the diversity of the IGS1 sequences exhibited by *T. asahii* strains obtained from Japan, the United States, and Brazil.³⁸ Rodriguez-Tudela et al also evaluated sequence polymorphisms of the IGS1 regions of 18 *T. asahii* strains and recognized six different genotypes from patients in Argentina, Brazil, and Spain. The majority of them were representative of genotypes 1 and 5.³⁹ Chagas-Neto et al analyzed the genotype distribution of 15 *T. asahii* isolates obtained from blood samples from patients in Brazil and found that the majority of these strains belonged to genotype 1 (86.7%).³ Kalkanci et al reported that from among 87 *T. asahii* clinical isolates from Turkey, as many as 69 strains (79.3%) represented genotype 1, followed by seven strains (8.0%) representing genotype 5, six strains (6.9%) representing genotype 3, three strains (3.4%) representing genotype 6, and only one strain (1.1%) representing genotype 4.¹⁹ Mekha et al studied 101 *T. asahii* strains in Thailand and ascertained that genotype 1 (45 strains, 44.5%) and genotype 3 (35 strains, 34.7%) were the predominant types, followed by genotype 7 (18 strains, 17.8%).⁴⁰ Sellami et al identified four IGS1 sequence types among the 28 *T. asahii* isolates that were studied, with majority of them being genotype 1 (46.4%) and genotype 4 (35.7%), followed by

genotype 7 (14.3%) and genotype 3 (3.6%) in Sfax, Tunisia.³⁷ A recent study by do Espírito Santo et al from Brazil identified 3 genotypes among the 12 *T. asahii* isolates, genotype 1 (41.7%), genotype 3 (33.3%), and genotype 5 (25%).³² A study by Rastogi et al in India on 20 *T. asahii* isolates recognized genotype III as the most common type, followed by genotype I and VII.⁴¹ Hence, we can see the influence of different geographical locations in the prevalence and predominance of a particular genotype. Although genotyping *Trichosporon* strains by sequencing the IGS regions is the most discriminative method as described in previous studies,^{19,35,38} not all clinical laboratories have sequencing options for routine analysis. Genotyping of strains can be done by RAPD analysis in laboratories using PCR techniques.

Pattern I was found to be the predominant pattern among the (34.2%) urine isolates and pattern X was the most predominant pattern among the blood (27.3%) and PCN (57.1%) isolates. Higher the correlation among the genotypes from a particular sample source, higher is the risk of exogenous nosocomial infection. Hence, typing of the strains can give informative data about the relatedness of the strains, especially if they are performed during an outbreak.

To conclude, molecular techniques enable rapid and reliable identification of *T. asahii* isolates at species level. Furthermore, this study reiterates the current outlook that *T. asahii* is the most common species associated with human clinical specimens and also has wide geographic distribution. Strain typing helps differentiate between the genotypes and reveals the correlation among them, establishing the relatedness of the strains, thus helping in outbreak investigation.

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3. Ethics Clearance: Institutional Ethical clearance—REF: IEC-NI/12/MAR/27/14

Conflict of Interest

None.

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