Genotype and Drug Susceptibility Analysis of *Trichosporon Asahii*

Running title: Analysis of a clinical fungus *Trichosporon asahii*

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Abstract: This study aims to explore the genotypes and drug susceptibility of *Trichosporon asahii* (*T. asahii*) to common antifungal drugs *in vitro*. Ten strains of *Trichosporon* were identified with API 20C AUX and Vitek 2 Compact according to morphology. Genotypes were identified by rDNA ITS, 26S rRNA PCR amplification, and sequence analysis. The minimum inhibitory concentration (MIC) of antifungal drugs was detected by E-test assay. The ten strains of *Trichosporon* were *T. asahii*. The strains were divided into three genotypes, namely, genotype IV (n = 7), genotype I (n = 2), and genotype III (n = 1), through sequence analysis. Susceptibility test showed that voriconazole and itraconazole, with MICs of 0.08 and 0.7 μg/ml, respectively, exhibited the best inhibitory activity against *T. asahii*. rDNA ITS sequence analysis can be utilized to identify *Trichosporon* strains. 26S rRNA IGS 1 genotyping can be utilized in epidemiological investigations. Voriconazole and itraconazole can be employed to treat *Trichosporon* infections because these drugs exhibit effective inhibitory activity against *Trichosporon*.
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Keywords: antifungal drugs; E-test susceptibility test; genotype; *Trichosporon asahii*

1. Introduction

The incidence of invasive fungal infections in critically ill patients, particularly in immunocompromised patients, continues to increase annually. These infections have elicited widespread global concern because of their severity and the resulting high mortality rate. Fungal infections include infections by common *Candida* and *Aspergillus* as well as by unusual opportunistic fungi, such as *Trichosporon*, *Fusarium*, and *Zygomycete*. *Trichosporon asahii* (*T. asahii*), a yeast-like fungus, is a member of *Trichosporon*. The fungus is an opportunistic pathogen present in the skin, respiratory tract, or gastrointestinal tract of immunocompromised patients and newborns. It can cause skin and lung infections as well as disseminated infections. Humans are often infected by *Trichosporon*ion through catheterization, drainage, burn wounds, or intestinal mucosa transplantation. Recent studies have reported that *T. asahii* infection is often accompanied by acute respiratory failure, renal failure, disseminated intravascular coagulation, and other symptoms; the infection is characterized by poor prognosis and high mortality (Chagas-Neto et al., 2009; Kalkanci et al., 2010; Guo et al., 2011; Menezes et al., 2012; Tuite and Lacey, 2013). Rapid and accurate identification of *Trichosporon* is important for the rational use of antifungal agents in clinics (Mekha et al., 2010; Sabharwal, 2010; Heslop et al., 2011; Wilke, 2011; Zhang et al., 2011; Pemán and Zaragoza, 2012; Tsai et al., 2012; Luong et al., 2013) because of the large difference in antibiogram between *Trichosporon* and common yeasts and among different kinds of *Trichosporon*. Current morphological and biochemical identification methods are based on fungal culture and do not involve rare fungi.

Studies on gene polymorphism and genotype have been conducted (Mekha et al., 2010; Pemán and Zaragoza, 2012). In the present study, ten strains of *Trichosporon* isolated from patients were identified and genotyped by morphological observation, biochemical identification (API 20C Aux/VITEK 2 Compact), and ITS (ITSl, 5.8S, ITS2) D1/D2 domain sequence analysis. E-test method was applied to determine the *in vitro* susceptibility of *Trichosporon* to five commonly used antifungal drugs. The objective is to provide a laboratory basis for the clinical selection of antifungal agents (Li et al., 2010; Tsai et al., 2012).

2. Materials and methods

2.1 Strains

Ten strains of *Trichosporon* isolated from the sputa of patients were provided by the Laboratory Medicine of the First Affiliated Hospital of the General Hospital of PLA, Beijing, China. The Ethics Committee of the hospital also provided their approval for the study. Written informed consent was obtained from the participants. *Candida parapsilosis* ATCC22019 from ATCC was used as the quality control strain, and *T. asahii* CBS2479 was used as the reference strain (Yang et al., 2012).

2.2 Identification

The fungi isolated from the sputa of patients

were cultured on SDA at 28 ℃ for 24 h to 28 h. After lactic acid phenol cotton blue (LPCB) staining, the fungi were observed under a microscope. Coded identification was then performed with API 20C AUX and Vitek 2 Compact.

2.3 PCR

Genomic DNA was extracted with a glass bead concussion. Specific primers were utilized for ITS1/ITS4, F63/R635, and 26SF/5SR to amplify the ITS, D1/D2, and IGS 1 regions, respectively. The respective product sizes were 595, 663, and 1110 bp. PCR was performed according to previously reported methods (Rodriguez-Tudela et al., 2007; Chagas-Neto et al., 2009; Kalkanci et al., 2010; Mekha et al., 2010; Guo et al., 2011). Two-way sequencing of the PCR products was performed by Shanghai Biological Engineering Company. The rDNA ITS sequences of *Trichosporon* were searched for in the GenBank sequence database.

2.4 Susceptibility test

A susceptibility test was performed according to CLSI methods (Clinical and Laboratory Standards Institute, 2008). Fresh colonies cultured on SDA at 28 ℃ for 24 and 48 h (those in slow growth were cultured for 48 h) were collected to formulate a 0.5 MCF suspension with 0.85% sterile saline. A sterile cotton swab was dipped in the fungi suspension and inoculated into an RPMI1640 solid medium by streaking. The surface was allowed to dry for 15 min. E-test strips were pasted onto the medium followed by incubation at 28 ℃ for 48 h to 72 h. MICs for fluconazol, voriconazole, itraconazole, and caspofungin were determined at 80% inhibitory rate, and MIC for amphotericin B was determined at 100% inhibitory rate.

3.1 Identification results

Sputum specimens were collected from patients with respiratory system diseases. The pathogenic fungi were isolated and identified with API 20C AUX or Vitek 2 Compact. The ITS sequence analysis results reveal that all 10 strains are *T. asahii*. Cladogram analysis of the 10 strains was performed (Figure 1).

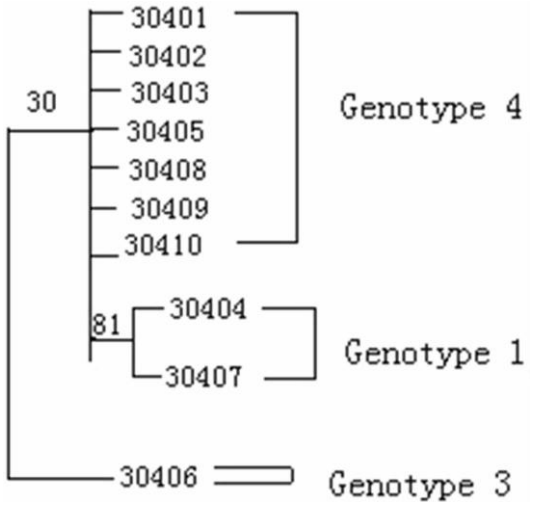

Figure 1 Cladogram analysis of ten strains of *Trichosporon* isolated from patients

3.2 typing

The 10 strains of fungi were successfully amplified, cloned, and sequenced. The reference strain belongs to genotype I. The ten strains isolated from patients belong to three genotypes, namely, genotype IV (n = 7, 70%), genotype I (n = 2, 20%), and genotype III (n = 1, 10%). The genotyping results are shown in Table 1.

3. Results

Table 1: ITS, D1/D2 and IGS 1 regions and their genotypes of ten strains of *Trichosporon* isolated from patients

Specimen number	*Sources of specimen*	*ITS*		*D1/D2*	
		GenBank No.	*Sequencing coincidence (%)*	*GenBank No.*	*Sequencing coincidence (%)*
30401	Department of Respiratory Medicine	FJ943429	(541/541) 100%	EU559350	(627/627) 100%
30402	Department of Respiratory Medicine	FJ943429	(541/541) 100%	EU559350	(627/627) 100%
30403	VIP department	FJ943429	(541/541) 100%	EU559350	(627/627) 100%
30404	Department of endocrinology	FJ943429	(540/541) 99.8%	EU559350	(627/627) 100%
30405	ICU	FJ943429	(541/541) 100%	EU559350	(627/627) 100%
30406	Department of Respiratory Medicine	FJ943429	(541/541) 100%	EU559350	(627/627) 100%
30407	Department of Respiratory Medicine	FJ943429	(541/541) 100%	EU559350	(627/627) 100%
30408	ICU	FJ943429	(541/541) 100%	EU559350	(627/627) 100%
30409	VIP department	FJ943429	(541/541) 100%	EU559350	(627/627) 100%
30410	ICU	FJ943429	(541/541) 100%	EU559350	(627/627) 100%

Table 1: ITS, D1/D2 and IGS 1 regions and their genotypes of ten strains of *Trichosporon* isolated from patients (continue)

Specimen number	*IGS1*		*Strain identified*	*IGS1genotype*
	GenBank No.	*Sequencing coincidence (%)*		
30401	FJ754250	(573/579) 99%	T.asahii	Genotype 4
30402	FJ754250	(573/579) 99%	T.asahii	Genotype 4
30403	FJ754250	(573/579) 99%	T.asahii	Genotype 4
30404	FJ754250	(579/579) 100%	T.asahii	Genotype 1
30405	FJ754250	(573/579) 99%	T.asahii	Genotype 4
30406	FJ754250	(567/584) 97.1%	T.asahii	Genotype 3
30407	FJ754250	(579/579) 100%	T.asahii	Genotype 1
30408	FJ754250	(573/579) 99%	T.asahii	Genotype 4
30409	FJ754250	(573/579) 99%	T.asahii	Genotype 4
30410	FJ754250	(573/579) 99%	T.asahii	Genotype 4

3.3 Susceptibility

Quality control strain *Candida parapsilosis* ATCC22019 was used to test the susceptibility of *T. asahii* to fluconazol, voriconazole, itraconazole, amphotericin B, and caspofungin. The MIC ranges and test results of the five antifungal agents against *Candida parapsilosis* ATCC22019 are listed in Table 2. The geometric means of the MIC of voriconazole and itraconazole against *T. asahii* are 0.08 and 0.7 μg/ml, respectively. However, the geometric means of the MIC of amphotericin B, fluconazol, and caspofungin are 1, 1.74, and 5.27 μg/ml, respectively. The MIC distribution of the 10 strains of fungi isolated from patients is listed in Table 3.

Table 2: The MIC ranges and test results of the five antifungal agents against the quality control strain *Candida parapsilosis* ATCC22019 (μg/ml)

Antifungal drugs	*MIC ranges*	*Test results*
Fluconazol	0.125-8	2
Voriconazole	0.016-0.064	0.032
Itraconazole	0.064-0.25	0.064
Amphotericin B	0.25-1	0.75
Caspofungin	0.25-2	1

Table 3: The MIC distribution of the ten strains of Fungi isolated from patients

Specimen number	*Type of specimen*	*Gender/ age*	*MIC values (μg/ml)*				
			Fluconazol	*Voriconazole*	*Itraconazole*	*Amphotericin B*	*Caspofungin*
CBS2479	Unknown	Unknown	1	0.032	0.5	1	4
30401	Sputum	Male/60	0.5	0.064	0.25	1	2
30402	Sputum	Male/54	2	0.064	0.25	1	1
30403	Sputum	Male/78	1	0.064	1	1	4
30404	Sputum	Female/63	8	0.064	1	1	8
30405	Sputum	Male/80	2	0.064	1	1	8
30406	Sputum	Male/74	1	0.5	1	1	8
30407	Sputum	Male/75	2	0.064	1	1	8
30408	Sputum	Male/75	2	0.064	0.5	1	8
30409	Sputum	Female/85	2	0.064	1	1	8
30410	Sputum	Male/80	2	0.064	1	1	8

4. Discussion

The incidence of piedra has exhibited a clear ascending trend in recent years (Chagas-Neto et al., 2009; Mekha et al., 2010; Sabharwal, 2010; Heslop et al., 2011; Kudo et al., 2011; Zhang et al., 2011; Menezes et al., 2012; Tuite and Lacey, 2013). The trend is related to the increasing incidence of malignant tumor, the increasing popularity of organ transplant, immune suppressive therapy, and chemotherapy, and the widespread use of broad-spectrum antibiotics and invasive medical operation. Fourteen strains of *Trichosporon* that cause infections in humans have been identified. Among these strains, *T. asahii* is the main pathogenic fungus in piedra and infections by non-Candida fungi (Ruan et al., 2009; Wilke et al., 2011; Pemán and Zaragoza, 2012; Luong et al., 2013). Therefore, attention should be focused on the understanding of *Trichosporon* infections and their treatment. However, distinguishing them from other yeasts based on morphology is difficult. Morphology or

coded identification method (API20C AUX or Vitek 2 Compact) generally results in failure in detection (Cejudo et al., 2010). Thus, the present study employed morphology, coded identification method, and molecular sequencing method (PCR amplification and sequence analysis of rDNA ITS, 26 s rRNA of D1/D2, and gene interval sequence IGS1 region) (Rodriguez-Tudela et al., 2007; Chagas-Neto et al., 2009; Kalkanci et al., 2010; Guo et al., 2010; Mekha et al., 2010) to identify and typify the piedra gene clinically. Results show that the identification accuracy of API20C AUX and Vitek 2 Compact are 91.7% and 75%, respectively. Sequence analysis of the rDNA ITS region at a sensitivity rate of 100% was rapid and accurate and can thus be used in clinical laboratories to assist in the identification of undistinguished *Trichosporon*.

The 10 strains of *T. asahii* isolated from patients were divided into three genotypes based on the sequence analysis of the IGS1 region. Seven strains belonging to genotype IV, a predominant type, were from specimens from the ICU (n = 3), VIP Department (n = 2), and the Department of Respiratory Medicine (n = 2). Two strains belonging to genotype I were from the Department of Respiratory Medicine (n = 1) and the Department of Endocrinology (n = 1). One strain belonging to genotype III was from the Department of Respiratory Medicine (n = 1). Analysis indicates that the popular genotype in Japan, Europe, and South America is genotype I (57% to 87%), whereas the popular genotype in America is genotype III (approximately 60%) (Chagas-Neto et al., 2009; Kalkanci et al., 2010; Guo et al., 2010; Mekha et al., 2010). These observations indicate that the genotypes in the IGS1 region of *T. asahii* differ across countries, regions, samples, environments, and positions. The genetic and biological characteristics are different as well.

The results of the susceptibility test *in vitro* show that voriconazole and itraconazole have effective antifungal activities against *T. asahii*. The geometric means of MIC against *T. asahii* are 0.08 and 0.7 μg/ml. The geometric mean of the MIC of amphotericin B against the ten *T. asahii* strains is 1 μg/ml. The MIC range of fluconazol against *T. asahii* is 0.5 μg/ml to 8 μg/ml, and the geometric mean of MIC is 1.74 μg/ml. The mean is higher than that of Brazil (1.1 μg/ml) (Menezes et al., 2012) but lower than that of Turkey (12.5 μg/ml) (Kalkanci et al., 2010). The MIC range of caspofungin against *T. asahii* was 1 μg/ml to 8 μg/ml, and its geometric mean of MIC is 5.27 μg/ml. These results suggest that the antibiotics sensitivity spectrum of *T. asahii* is different from that of other clinically common yeast (Ruan et al., 2009). Different kinds of fungi exhibit different antifungal susceptibility. Thus, identifying the fungus as accurately as possible to the levels of genus and species is significant in selecting a clinical antifungal.

The susceptibility of 10 strains of *T. asahii* to fluconazol, voriconazole, itraconazole, amphotericin B, and caspofungin was detected through E-test method in this study. *Candida parapsilosis* (ATCC 22019) was used as the quality control strain; its MIC was within the allowable standard range. However, the current drug susceptibility test M27-A3 schema (Clinical and Laboratory Standards Institute, 2008) for yeast has no break point for *Trichosporon*. Thus, in this study, the geometric means of MIC were derived to reflect the difference in the performance of antifungal agents against *T. asahii* as well as to provide a reference for the determination of a break point in the future.

With the increase in the number of new, uncommon opportunistic fungal infections, traditional identification methods (such as morphological and biochemical reactions) for yeast have become unable to meet clinical requirements. Sequence analysis by PCR amplification of the ITS or IGS1 regions of ribosomal rDNA with fungal universal primers (Al-Mahmeed et al., 2009; Zeng et al., 2009) can rapidly and accurately identify the pathogens of infection. The IGS1 region is more variable than the ITS region in *T. asahii* strains, which can be of great significance in gene polymorphism and genotyping of *T. asahii* as well as in research on epidemiological investigation, monitoring, distributing feature, and anti-infective therapy. The antibiotics sensitivity spectrum of *T. asahii* is different from those of other clinically common yeasts. Therefore, a fungal susceptibility test should be performed prior to the clinical usage of effective antifungal drugs to improve the cure rate of fungal infections.

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