Trichosporon asahii causing nosocomial urinary tract infections in intensive care unit patients: genotypes, virulence factors and antifungal susceptibility testing

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Trichosporon asahii is the causative agent of both superficial and deep-seated infections of increasing morbidity and mortality. Urinary tract infections (UTIs) due to T. asahii, frequently associated with indwelling medical devices, have been reported over the years. However, few studies have specifically focused on the genotypic diversity of T. asahii isolates from urine specimens from intensive care units (ICUs), let alone potential virulence factors and antifungal susceptibility testing. In the present study, 23 T. asahii isolates were collected from UTI patients in ICUs between January 2008 and January 2012. Three genotypes (I, III, IV) were determined based on the combination of internal transcribed spacer and intergenic spacer locus PCR. Protease, phospholipase and haemolysin production was assessed by halo formation on corresponding agar plates. Only haemolytic activity was observed to varying degrees. Neither protease nor phospholipase was detectable. Biofilm formation on polystyrene surfaces was detected through a formazan salt reduction assay. All clinical isolates had the ability to form biofilm. In contrast to the susceptibility of planktonic T. asahii cells to clinically used amphotericin B, 5-flucytosine, fluconazole, itraconazole and voriconazole, a remarkable rise in the MICs of these for biofilm T. asahii cells was observed. Our results suggested that genotype IV was the most prevalent genotype among T. asahii isolates from ICUs in China. Haemolysin and biofilm might contribute to the pathogenicity and recurrence of T. asahii-related UTIs. Although triazoles, especially voriconazole, were effective against planktonic T. asahii cells, they failed to treat preformed biofilms.

INTRODUCTION

Urinary tract infections (UTIs) caused by fungi, frequently associated with medical devices, have increased and cause great morbidity and mortality among nosocomial patients (Frank et al., 2009; Kauffman et al., 2000). Although Candida albicans is still being isolated predominantly, the prevalence of Trichosporon has increased greatly (Kumar et al., 2011; Sabharwal, 2010; Sood et al., 2006). Trichosporon species are emerging fungal pathogens capable of causing localized or systemic infections, especially in immunocompromised patients with cancer, haematological diseases or organ transplantation (Mirza, 1993; Walsh et al., 2004). Trichosporonosis has also been found in non-immunocompromised patients, including patients with prosthetic valves, underlying peritoneal dialysis and intravenous and urinary catheters (Krzossok et al., 2004; Martinez-Lacasa et al., 1991; Mooty et al., 2001; Rastogi & Nirwan, 2007; Sood et al., 2006). Of note, Trichosporon asahii is the major cause of disseminated or deep-seated trichosporonosis (Chagas-Neto et al., 2009; Girmenia et al., 2005).

Other reports (Lundstrom & Sobel, 2001; Richards et al., 2000; Sugita et al., 2001) have suggested that genetic diversities and biochemical characteristics of T. asahii strains are related to the specimen sources as well as hospitalized settings, being most common in intensive care units (ICUs). However, all previous studies generally included a diversity of clinical sources, such as skin, sputum, urine and blood (Chagas-Neto et al., 2009; Marty et al., 2003; Rodriguez-Tudela et al., 2007; Yun et al., 2006). There have been no such studies that target the genotypic and pathogenic attributes as well as susceptibility testing of specified T. asahii isolates, such as those from elderly ICU patients with UTIs.

The first objective of this study was to identify the prevalent genotype of T. asahii isolates from UTI patients by virtue of
intergenic spacer (IGS1) locus PCR. The second objective was to gain information about the potential virulence factors (protease, phospholipase, haemolysin, biofilm). The third objective was to evaluate the in vitro susceptibilities of T. asahii cells under both planktonic and biofilm growth conditions against five commonly used antifungal agents.

**METHODS**

**Micro-organisms.** The present study was conducted at Beijing Friendship Hospital, a tertiary A level, general hospital. A collection of 23 T. asahii strains from urine specimens from all patients undergoing fungal culture from January 2008 to January 2012 were evaluated. Basic information on patients with positive culture results was extracted from the hospital Microbiology Department database. Strains were preserved in sterile freezing tubes at −80 °C until use.

**Inclusion criteria and definitions.** Funguria was defined according to previous reports with appropriate modifications (Lundstrom & Sobel, 2001; Sugita et al., 2001). The presence of ≥ 10000 c.f.u. ml⁻¹ of any fungal species in the urine was regarded as funguria. Persistence of funguria was defined as at least two consecutive urine cultures positive for fungus. A second urine culture without evidence of fungus was indicative of clearance.

**Exclusion criteria.** Patients who had already been diagnosed with funguria before being admitted to ICUs or with duration time in ICUs ≤ 48 h, and those patients who died within 1 week of admission to hospital, were excluded. Repeat specimens from the same patient within a 1 week period were excluded. Colony counts <10000 c.f.u. ml⁻¹ were not included in the statistics.

**Selection and identification of clinical isolates.** Quantitative urine cultures were performed referring to standard laboratory methods. Following the manufacturer’s guidelines, clinical strains were cultured in YEPD medium (1 % yeast extract, 2 % peptone, 2 % glucose) with 150 r.p.m. horizontal shaking for 24–48 h at 35 °C. T. asahii strains were first identified via VITEK-2 Compact with YST cards, and then verified through DNA sequencing of the internal transcribed spacer (ITS) locus of the rDNA, using primers ITS1/ITS4 (ITS1: 5'-TCCTTGGTATGGTGAACCTGCGG-3'; ITS4: 5'-TCTTCCGCT- TTATGATATGC-3') (Ahmad et al., 2004).

**Sequencing of the IGS1 locus and genotyping analysis.** Extraction of DNA from the T. asahii isolates was carried out according to a previous report (Makimura et al., 1994). DNA segments containing the IGS1 locus were amplified using primers 26sF (5'-ATCCTTTGCCACAGCCCTTGA-3') and 5SR (5'-AGCTTGAACCTGCGCAGATC-3') (Guo et al., 2011). Purified PCR products of the isolates were sequenced by Invitrogen, and the IGS1 sequence asahii was aligned with 12 previously identified T. asahii isolates by Invitrogen, and the IGS1 sequence asahii was sequenced by Invitrogen, and the IGS1 sequence was deposited in GenBank.

**Virulence factors.** To determine the possible virulence factors, three assays were carried out for each isolate on separate occasions, each assay in triplicate. The mean values were used for analysis.

**Protease and phospholipase production.** With reference to C. albicans (Furlaneto-Maia et al., 2008; Negri et al., 2010), protease and phospholipase were detected by the formation of a precipitation halo around each colony due to degradation. An aliquot (5 µl) of a 1 x 10⁸ c.f.u. ml⁻¹ suspension was resuspended in saline (1 ml) and spot-inoculated on 0.2 % BSA agar plates (pH 5.0) and 8 % egg yolk agar plates. Another 5 µl saline without yeast cells was spot-inoculated simultaneously as a blank control. The plates were then incubated at 35 °C for 7 days. Enzymic activity (P) was determined by the ratio between the colony diameter and the colony diameter plus the precipitation zone diameter, as described previously (Dagdeviren et al., 2005; Price et al., 1982). C. albicans ATCC 10231 and ATCC 2512 were used as positive and negative control, respectively.

**Haemolytic activity.** Haemolytic activity was evaluated using a plate assay described previously with a slight modification (Luo et al., 2001). Freshly cultured colonies of T. asahii were prepared in saline solution at 10⁶ c.f.u. ml⁻¹. Five microlitres of this suspension was spot-inoculated on sugar-enriched sheep blood plates. Another 5 µl saline without yeast cells was spot-inoculated as a blank control. The plates were incubated at 35 °C in 5 % CO₂ for 96 h. The presence of a translucent halo around the inoculation site, viewed with transmitted light, indicated positive haemolytic activity (H₀). H₀ expressed the ratio of the colony diameter to the diameter of the translucent zone of haemolysis (mm) (Furlaneto-Maia et al., 2008; Negri et al., 2010). C. albicans ATCC 90028 and ATCC 2201 were used as positive and negative control, respectively.

**Biofilm formation.** Biofilm formation was assayed colorimetrically based on sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium] -bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) reduction as previously reported (Di Bonaventura et al., 2006). Briefly, T. asahii isolates grown overnight on Sabouraud dextrose agar plates at 35 °C were harvested, washed once with sterile PBS and then resuspended in RPMI 1640 (Sigma Aldrich; R8758) adjusted to pH 7.0 with 0.165 M MOPS (Sigma Aldrich; 1132-61-2) to a concentration of 10⁵ c.f.u. ml⁻¹. Biofilms were formed by pipetting 2 ml of the standardized cell suspension into commercially available presterilized, polystyrene, 35 mm-diameter tissue culture Petri dishes (Costar) and incubated at 35 °C. After 60 min of incubation (adhesion phase), dishes were washed twice with sterile PBS to remove non-adherent cells and then refilled with 2 ml fresh RPMI 1640-MOPS medium. Samples were then incubated for up to 48 h at 35 °C, replacing the medium every 24 h of incubation (biofilm formation phase). At the end of the incubation time, the medium was aspirated and non-adherent cells were removed by washing the biofilms twice in 2 ml sterile PBS. As a control, additional dishes were processed in an identical fashion, except that no T. asahii cells were added. Of note, XTT was prepared in RPMI 1640-MOPS at 0.5 mg ml⁻¹, and then the electron-coupling agent phenazine methosulfate (PMS) was added to a final concentration of 12.5 µM. A 1.5 ml aliquot of the XTT-PMS solution was added to each pre-washed biofilm and to control wells (for the measurement of background XTT reduction levels). The plates were then incubated in the dark for 2 h at 35 °C. Supernatants were centrifuged and then divided into aliquots in a 96-well microplate for reading. A colorimetric change in the XTT reduction assay, which is a direct correlation of the metabolic activity of cells within the biofilm, was then measured in a microtitre plate reader (TECAN; Infinite 200) at 492/630 nm (read/reference).

**In vitro antifungal susceptibility testing.** For consistency with biofilm cells, the antifungal susceptibility testing of planktonic cells was assessed using the CLSI M27-A3 method (CLSI, 2009) as well as an XTT-based colorimetric method described previously with minor modifications (Hawser et al., 1998). Amphotericin B (Sigma-Aldrich; 1397-89-3), fluconazole (Sigma-Aldrich; 2022-85-7), fluconazole (Sigma-Aldrich; 86386-73-4), iraconazole (Sigma-Aldrich; 84625-61-6) and voriconazole (Sigma-Aldrich; 137234-62-9), prepared in RPMI 1640-MOPS, were used in this study. Briefly, the susceptibility plates with antifungal agents (at twofold concentration gradients) were prepared as described in the M27-A3 procedures for the susceptibility testing of yeasts (CLSI, 2009). Two hours prior to the end point reading, plates were agitated, and 50 µl of a mixture of XTT (0.5 mg ml⁻¹) plus PMS (12.5 µM) was added to all wells. Plates were incubated for 2 h at 35 °C for colour development. Because a
previous report (Hawser et al., 1998) identified that 100 % reduction in metabolic activity is equivalent to the MIC as determined by the CLSI M27-A3 method for planktonic yeast cells, MICS (MICXTT) were determined spectrophotometrically [492 nm/630 nm (read/reference)] as the lowest concentration of antifungal agent causing a 100 % reduction in metabolic activity (no colour change occurring).

For antifungal susceptibility testing of biofilm cells, antifungal agents at concentrations ranging from 16 to 1024 µg ml⁻¹, prepared in fresh RPMI 1640-MOPS, were assayed against 48 h biofilms preformed on 35 mm-diameter tissue culture polystyrene dishes (Costar). Following incubation, biofilms were washed twice with sterile PBS and then exposed to each tested antifungal agent for another 24 h at 37 °C. A series of antifungal agent-free dishes were included as growth controls. The effect of antifungal agents against biofilms was measured using the XTT reduction assay described above for quantifying biofilm formation. The antifungal concentrations which caused a 100 % reduction in metabolic activity (MICXTT) of biofilms were determined.

Statistical analyses. Statistical analyses were performed using SPSS 11.5 statistical software. All data were expressed as means ± SEM, and were analysed using Student's t-test for comparisons between two groups, and one-way ANOVA followed by Fisher's least significant difference (LSD) test for multiple groups. Statistically significant differences were considered for values with *P<0.05 and **P<0.01.

RESULTS

Demographic and clinical data

Twenty-three patients with positive culture results for T. asahii in three consecutive cultivations were included from all hospitalized inpatients who underwent urine fungal culture. Only strains isolated before antifungal therapy were analysed for each patient.

All patients in this study were hospitalized in ICUs and 74 % of the episodes affected males. The median age of patients was 80 years old (ranging from 75 to 91 years old). Receipt of systemic antibiotics was the most commonly recognized risk factor. A urinary drainage device was present in 60.8 % of patients prior to or during funguria. All the patients suffered from various underlying conditions, such as diabetes, hypertension and chronic disease. However, none of the patients had received immunosuppressive therapy and none of the 23 patients had any urological malignancy or malformation. More detailed information is shown in Table 1.

Molecular identification and genotyping of T. asahii isolates

For accurate identification of these isolates, we employed diagnostic PCR with the primer pair ITS1/ITS4 soon after VITEK-2 verification. DNA bands of 520 bp were obtained from all strains tested, and also from the Trichosporon reference strain (CBS 2479). PCR products were aligned with the genome database in GenBank and identified as T. asahii.

For further intraspecies identification, DNA fragments from the IGS1 region were amplified with the primer pair 26sF/5sR, and the 642 bp products were sequenced and aligned with already known genotypes in the GenBank database. Among the 23 T. asahii isolates, the predominant genotype was type IV (52.2 % = 12/23), followed by type III (30.4 % = 7/23) and type I (17.4 % = 4/23).

Protease and phospholipase production

After 7 days incubation on specified BSA or egg yolk agar plates, no precipitation zones around colonies of any of the isolates were observed. Enzymic activity (Pz) values of 1.00 and 1.00, respectively, indicated that neither protease nor phospholipase activity was detectable among all T. asahii isolates of different genotypes.

In vitro haemolysin production and biofilm formation

To establish optimal conditions for T. asahii biofilm formation on polystyrene surfaces, we carried out several preliminary experiments on inoculum size (10⁴, 10⁵ or 10⁶ c.f.u. ml⁻¹), adhesion time (30, 60 or 120 min) and biofilm formation time (24, 48 or 72 h). Finally, we selected an inoculum size of 10⁵ c.f.u. ml⁻¹, an adhesion time of 60 min and a biofilm formation time of 72 h as optimal experimental conditions for culturing T. asahii biofilms on polystyrene surfaces, which would yield the best correlation with the XTT assay. The biofilm formation abilities of the 23 clinical isolates were illustrated as absorbance readings (A492/630) (Fig. 1a) from 0.05 to 1.06. Mean haemolytic activity (Hz) values, ranging from 1.06 to 1.44, were detected after 96 h incubation on blood agar plates (Fig. 1b).

Susceptibility testing of planktonic and biofilm cells to antifungal agents

In vitro antifungal susceptibilities of planktonic and biofilm cells of T. asahii to amphotericin B, 5-flucytosine, fluconazole, itraconazole and voriconazole are summarized in Table 2. For planktonic cells, MICS determined by both the CLSI and XTT methods revealed that voriconazole was the most active agent against all tested isolates (MIC 0.06 µg ml⁻¹). However, amphotericin B (MIC 0.50–0.79 µg ml⁻¹), 5-flucytosine (MIC 3.75–4.57 µg ml⁻¹), fluconazole (MIC 1.27–1.63 µg ml⁻¹) and itraconazole (MIC 0.11–0.13 µg ml⁻¹) were relatively poor in activity.

The in vitro activities of the tested antifungal agents against preformed T. asahii biofilms were determined by the XTT assay (Table 2). The data revealed that, although the tested antifungal agents were active against planktonic T. asahii cells, preformed biofilms were resistant to all the antifungal agents, even at a concentration up to 1024 µg ml⁻¹.

DISCUSSION

Trichosporon species are medically important yeast pathogens, frequently associated with indwelling medical devices (Moretti-Branchini et al., 2001; Pini et al., 2005). In spite of
Table 1. Microbiological and clinical data for 23 episodes of *T. asahii* UTIs

All patients were diagnosed with funguria but without fungaemia, and all received antibiotic therapy.

<table>
<thead>
<tr>
<th>Reference strain and patient</th>
<th><em>T. asahii</em> genotype</th>
<th>Gender of patient*</th>
<th>Age of patient</th>
<th>Underlying diseases</th>
<th>Urinary catheter</th>
<th>Other infections</th>
<th>Antifungal therapy†</th>
<th>Clinical outcome</th>
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<td>1</td>
<td>I</td>
<td>M</td>
<td>75</td>
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<td>NA</td>
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<td>2</td>
<td>I</td>
<td>M</td>
<td>78</td>
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<td>NA</td>
<td>NA</td>
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<td>I</td>
<td>M</td>
<td>80</td>
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<td>NA</td>
<td>FLC + VRC</td>
<td>Discharged</td>
</tr>
<tr>
<td>4</td>
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<td>F</td>
<td>77</td>
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<td>No</td>
<td>NA</td>
<td>NA</td>
<td>Discharged</td>
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<td>5</td>
<td>III</td>
<td>F</td>
<td>82</td>
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<td>FLC</td>
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<td>6</td>
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<td>M</td>
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<td>Death</td>
</tr>
<tr>
<td>7</td>
<td>III</td>
<td>M</td>
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<td>Chronic obstructive pulmonary disease</td>
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<td>NA</td>
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<td>8</td>
<td>III</td>
<td>M</td>
<td>79</td>
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<td>NA</td>
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<td>9</td>
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<td>FLC + VRC</td>
<td>Discharged</td>
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<tr>
<td>12</td>
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<td>M</td>
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<td>Cardiac failure</td>
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<td>NA</td>
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NA, Not available.

*M, Male; F, female.
†FLC, Fluconazole; VRC, voriconazole; AMB, amphotericin B.
their in vitro sensitivity to commonly used antifungal agents, persistent infection or recurrence of trichosporonosis after treatment is a common phenomenon (Walsh et al., 2004).

Consistent with previous studies (Colombo et al., 2011; Padhye et al., 2002; Rodriguez-Tudela et al., 2007), there were susceptible populations commonly associated with trichosporonosis in this study (Table 1). All 23 patients were from ICUs and elderly (≥ 75 years). All had antibiotic treatment, and 60.8% (14/23) of patients underwent urinary catheterization. All patients had various underlying diseases. Diabetes was the most common one, accounting for 34.7%, followed by hypertension (30.4%).

Relevant laboratory examination results from the UTI patients gave us some important information on the immediate response of the hosts against T. asahii infection (increased white blood cells in both blood and urine, etc.) and some risk factors for T. asahii funguria (higher blood glucose, lower total protein and albumin level, etc.). Therefore, when these clinical indicators appear with obscure infections, other than caused by bacteria, a fungal cause should be considered.

In the present study, we made use of the IGS1 locus to provide discriminatory information for distinguishing between phylogenetically closely related strains of T. asahii (Sugita et al., 2002). IGS1 sequence analysis also shows great potential as an epidemiological tool. For instance, the geographical distribution of different genotypes of T. asahii isolates by IGS1 sequencing has been described in a number of studies (Kalkanci et al., 2010; Rodriguez-Tudela et al., 2007; Sugita et al., 2001). Up to now, IGS1 sequencing has led to the identification of 12 genotypes, genotypes I–V from the United States and Japan, genotype VI from Spain, Argentina and Brazil, genotypes VII–IX from Thailand and Turkey and three novel genotypes, X, XI and XII (Xia et al., 2012), through maximum-parsimony analysis of the IGS1 sequences. Consistent with a previous report (Guo et al., 2011), genotype IV was the most common genotype (52.2%) among the 23 UTI-related T. asahii isolates in China, but has only rarely been found (1.0–4.5%) in Japan, South America and Turkey (Chagas-Neto et al., 2009; Kalkanci et al., 2010; Sugita et al., 2002). The other two genotypes in this study were genotype III and genotype I, accounting for 30.4% and 17.4%, respectively. In contrast, genotype I has been reported to be common in the United States (about 60%) and the most prevalent type in Japan, South America and Europe (57–87%) (Chagas-Neto et al., 2009; Kalkanci et al., 2010; Rodriguez-Tudela et al., 2007; Sugita et al., 2002).

The infective ability of yeasts depends on specific virulence mechanisms conferring the ability to colonize mucosal or synthetic surfaces and to invade host tissues by disrupting host-cell membranes. In contrast to the extensive studies regarding virulence of C. albicans, few similar investigations of T. asahii isolates have been reported. We attempted to detect several virulence factors as for C. albicans with slight modifications (Dagdeviren et al., 2005; Furlaneto-Maia et al., 2008). All isolates presented haemolytic activity to different degrees after 96 h incubation, longer than for C. albicans (48 h). All strains tested in this study were able to form biofilms on polystyrene surfaces. This phenomenon has important clinical implications for therapy of biofilm-associated infection, which is difficult to treat and recurs easily (Shin et al., 2002). Of note, although 52.2% (12/23) of all T. asahii isolates were type IV, type III seems to be more pathogenic with higher haemolytic and biofilm formation ability (Fig. 1).

Susceptibility testing results showed that all clinical isolates exhibited low MICs of amphotericin B (0.5 µg ml⁻¹), in contrast to other studies where T. asahii strains had low susceptibility to amphotericin B (Chagas-Neto et al., 2009; Kalkanci et al., 2010; Rodriguez-Tudela et al., 2005). Fluconazole was less active than itraconazole and voriconazole, with higher geometric mean MICs (Table 2). Voriconazole in vitro was the most effective agent against planktonic T. asahii (geometric mean 0.06 µg ml⁻¹), as reported previously for T. asahii isolates (Mekha et al., 2010; Rodriguez-Tudela et al., 2005). In this study, we did not choose the newer antifungal agents echinocandins, because Trichosporon is intrinsically resistant to echinocandins. Breakout in patients receiving echinocandin prophylaxis has been reported (Bayramoglu et al., 2008; Fera et al., 2009). On the whole, consistent with a previous report (Wolf et al., 2001), the triazole antifungal agents
exhibited greater in vitro activities against planktonic *T. asahii*. Furthermore, due to the poor physiological condition of severely ill patients in ICUs, a potent agent with a much broader antifungal activity spectrum, such as voriconazole, may be recommended.

The lack of clear and specific indications for the clinical interpretation of *T. asahii* UTI for clinicians is still a problem. Further collection and study of *T. asahii* UTI isolates from ICU patients in China are ongoing, in the hope that the nationwide surveillance programme CHIF-NET will provide enough clinical and epidemiological information for thorough and in-depth investigations in the near future.

In conclusion, the present study provided accurate species identification and genotyping of *T. asahii* UTI isolates by combining the use of ITS and IGS1 sequencing. Three genotypes were identified: I, III and IV. Genotyping analysis by IGS1 sequencing suggested that genotype IV was the predominant genotype. However, genotype III might be more pathogenic because of its higher haemolytic and biofilm formation activities. Among the agents tested against *T. asahii* isolates, voriconazole in vitro was the most effective for cells in planktonic form, but was ineffective for those in biofilm form.

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