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ARTICLE

Emerging *Trichosporon asahii* in elderly patients: epidemiological and molecular analysis by the DiversiLab system

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Abstract *Trichosporon asahii* has been recognized as an emerging opportunistic agent for invasive infections, mainly in immunocompromised patients. Urinary tract infections by this pathogen may also occur, especially in patients with urinary obstruction or those undergoing vesical catheterization and antibiotic treatment. Many outbreaks of *Trichosporon* spp. have been detected after urinary catheter manipulations. We report the molecular–epidemiological characterization of *T. asahii* in our institution using the DiversiLab system for the molecular strain typing and compare three different methods for susceptibility testing. Our results present *T. asahii* as an emergent pathogen in elderly patients with urinary drainage devices that can be adequately treated with triazoles, with voriconazole being the most active. Broth dilution and Vitek 2 had good concordance, while Etest showed more discrepancies. In addition, the DiversiLab system for clonal strain typing may be a useful tool for fast and accurate management of nosocomial outbreaks.

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Introduction

Trichosporon species are widely distributed in nature and can be part of the normal flora of the human skin and gastrointestinal tract [1]. Approximately 11 % of healthy men and up to 14 % of women are colonized by this microorganism on their perigenital skin [2]. Although many clinical isolates of this fungus are associated to colonization or superficial infection, *Trichosporon* has been recognized as an emerging opportunistic agent for invasive infections, mainly in immunocompromised patients (cancer, hematological diseases, or organ transplantation) [3]. Urinary tract infections by this pathogen may also occur, especially in patients with urinary obstruction or those undergoing vesical catheterization and antibiotic treatment [2]. In addition, it has also been found in immunocompetent individuals, including patients with prosthetic valves, underlying peritoneal dialysis, and intravenous or urinary catheters [4]. *Trichosporon asahii* is the most common in disseminated infections and is characterized by its relatively low susceptibility to amphotericin B. Recently, azole-resistant *T. asahii* strains have been isolated from patients [5].

Many outbreaks of *Trichosporon* spp. have been detected after urinary catheter manipulations. Other reports [6] have suggested that the genetic diversities of *T. asahii* strains are related to the specimen sources as well as hospitalized settings, being the most common in intensive care units. Although deep-seated trichosporonosis is an emerging mycosis with high mortality, information on its causatives is still limited.

In order to understand the emergence of *T. asahii* in elderly patients from our institution, the characteristics of the strains of this pathogen isolated during the last two years were investigated. Characterization was based on the molecular identification, drug susceptibility testing comparing different methods, and genotyping using the sequence analysis of the ribosomal DNA intergenic transcribed spacer 1 (IGS1)

regions and automated repetitive-sequence-based polymerase chain reaction (rep-PCR) (DiversiLab system, bioMérieux, Marcy l'Etoile, France).

Materials and methods

Patients and fungal isolates

From April 2010 through December 2012, a total of 32 hospitalized patients were infected or colonized by *T. asahii*. The isolates were recovered at the University Hospital Complex of Santiago de Compostela (Spain), a 1,120-bed teaching hospital in the northwest of Spain. Only one isolate per patient was used in this study, and all of them were recovered from urine samples. Only one patient had *T. asahii* in other body locations (peritoneal fluid).

Species identification

Species identification of isolated fungi was performed by biochemical tests using the Vitek 2 system (bioMérieux, Marcy l'Etoile, France) and by molecular procedures described by Rodriguez-Tudela et al. based on PCR and DNA sequencing of the internal transcribed spacer (ITS) and IGS regions of the rRNA genes [7].

Antifungal susceptibility testing

Minimum inhibitory concentrations (MICs) were determined in duplicate assays with three different methods: (1) Vitek 2 system (bioMérieux, Marcy l'Etoile, France), according to the manufacturer's instructions; (2) Etest (bioMérieux, Marcy l'Etoile, France), following the manufacturer's recommendations. Briefly, an inoculum concentration of $1–5\times10^6$ cell/mL was poured onto RPMI 1640 agar with 2 % glucose plates. The plates were incubated at 35° C and read after 24 and 48 h. And (3) broth dilution according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) method [8].

Molecular strain typing

Genotyping of 27 of the isolates was performed by two different methods (five isolates were not available for molecular studies):

1. The DiversiLab Fungal DNA Fingerprinting Kit was used for rep-PCR amplification of non-coding intergenic repetitive elements in the genomic DNA of *T. asahii*. The amplicons were analyzed using the DiversiLab system (bioMérieux, Marcy l'Etoile, France). Data analysis was performed with web-based software using the Pearson and Kullback–Leibler coefficient to determine distance matrices and the unweighted pair group method with arithmetic mean (UPGMA) to create dendrograms. Reports were automatically generated and included in the dendrogram, electropherograms, virtual gel images, scatter plots, and selectable demographic fields to aid the interpretation of the data. Sample relationships for rep-PCR were designated as follows: indistinguishable: >97 % similarity and no band differences; similar: >95 % similarity and one to two band differences; and different: <95 % similarity and three or more band differences, as done by other authors [9].
2. Amplification by PCR and DNA sequencing of the IGS1 regions of the rRNA genes as described previously [10]. The sequences obtained were compared to the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/), as well as with the database from the Department of Mycology of the Spanish National Centre for Microbiology (Majadahonda, Madrid), which holds 5,000 sequences from strains belonging to 270 different fungal species. This database was designed by the Spanish National Centre for Microbiology and has restricted access.

Results

Thirty-two patients with positive urine cultures were included from all hospitalized inpatients in the period of the study. All patients were hospitalized in the section of the internal medicine unit for old patients (geriatric unit), except for one who was in the critical care unit. The median age of the patients was 85 years old (ranging from 56 to 97 years old). During the years studied, a total of 4,858 patients were admitted to the geriatric unit, with a median age of 80 years. Urinary tract infection was suspected in 882 of them, resulting in positive urine cultures in 365 cases. *T. asahii* was recovered in 31 of them.

Antibiotic treatment and a urinary drainage device were the most commonly recognized risk factors present in all the studied patients (100 % of them). All the patients suffered from underlying conditions, mainly pneumonia (12/32), sepsis (8/32), and renal diseases (6/32). Nevertheless, none of them had received immunosuppressive therapy and only one had cancer. The majority of the patients had not received antifungal treatment, with fluconazole being the most frequent drug used in those that were treated. Some patients died (9/32), more likely due to their old age and severe underlying diseases (Table 1).

All fungal isolates were identified as *T. asahii* by both Vitek 2 and molecular methods [7].

Table 1 Clinical data for 32 patients with *Trichosporon asahii*

Strain	Patient	Date of collection	Gender	Age (years)	Underlying disease	Predisposing conditions	Clinical manifestation	Antifungal therapy	Clinical outcome
T1	M.V.V.	February 2010	M	65	Alcoholism	AT, PBC, PC	Fever, UTI	Voriconazole	Discharged
T2	R.C.A.	February 2011	M	92	Chronic kidney disease	AT, PC, UC	Fever	NO	Death
T3	M.V.R.	March 2011	M	95	Chronic kidney disease	AT, UC, PA, TS	Fever, UTI	NO	Discharged
T4	V.F.P.	March 2011	M	77	Chronic kidney disease	AT, PC, UC, TS, PS	UTI	NA	Discharged
T5	J.C.G.	March 2011	M	85	Chronic kidney disease, sepsis	AT, UC, PC, PA	Fever	NO	Death
T6	J.I.B.	March 2011	M	68	Sepsis	AT, UC, PC	Fever	NO	Discharged
T7*	C.L.S.	April 2011	F	86	Diabetes	AT, UC, CP, PA	Fever	NO	Discharged
T8	M.V.R.	April 2011	F	86	Sepsis, COPD	AT, PBC, PC	Fever, UTI	Fluconazole	Discharged
T9*	G.U.C.	June 2011	M	90	Nosocomial pneumonia	AT, PBC, PC	UTI	NO	Discharged
T10	J.L.S.R.	July 2011	M	58	Sepsis, ascites	AT, PBA, PC	Fever, UTI	Fluconazole	Death
T11	C.M.R.	July 2011	F	82	Sepsis	AT, PBC, CP, CC	Fever, UTI	Fluconazole	Discharged
T12	A.C.D.	July 2011	M	85	Dementia	AT, PBA, PC, PA	Fever	NO	Discharged
T13	J.S.M.	August 2011	M	92	COPD	AT, PBA, PC, PA	Fever, UTI	Fluconazole	Death
T14	A.V.L.	August 2011	M	85	Malnutrition, acute renal failure	AT, PBA, PC, PS	Fever	NO	Discharged
T15*	F.N.B.	August 2011	M	92	Nosocomial pneumonia	AT, PBA, PC, PA	Fever	NO	Discharged
T16*	J.V.M.	September 2011	M	94	Nosocomial pneumonia	AT, PBC, PC, PA	Fever	NO	Death
T17	O.A.P.I.	February 2012	M	90	Respiratory failure	AT, UC, PC, TS	Fever, UTI	NO	Death
T18	J.J.S.A.	February 2012	M	76	Bacteremia	AT, PBC, MV, PC, PA	UTI	Nistatin	Discharged
T19	M.C.N.	February 2012	M	56	Pneumonia	AT, PBC, PC, MV, PA	Fever	Fluconazole	Discharged
T20	M.J.G.S.	March 2012	F	92	Pneumonia	AT, PBC, PC, TS, PA	Fever, UTI	NO	Discharged
T21	A.G.S.	March 2012	M	81	Pneumonia, urinary sepsis	AT, PBC, PC	Fever	NO	Discharged
T22*	F.B.B.	March 2012	M	80	Neutropenia	AT, PBC, PC, PA	Fever, UTI	Voriconazole	Discharged
T23	M.C.P.G.	October 2012	F	91	Urinary sepsis	AT, PBC, PC, PA	Fever, UTI	NO	Discharged
T24	J.V.M.	October 2012	M	95	Nosocomial pneumonia	AT, PBC, PC, PA	UTI	NO	Death
T25	L.V.M.	November 2012	M	60	Nosocomial pneumonia, sepsis	AT, PBC, MV	UTI	Anidulafungin	Discharged
T26	J.L.P.	November 2012	M	79	Neutropenia	AT, PBC, PC, PA	UTI	VOR	Discharged
T27	S.C.V.	November 2012	M	92	Pneumonia	AT, PBC, PC, PA	UTI	NO	Discharged
T28	E.L.G.	November 2012	F	95	Pneumonia	AT, PBC, MV	UTI	NO	Death
T29	A.R.I.	November 2012	M	57	Acute renal failure	AT, UC	UTI	NO	Discharged
T30	M.F.M.	December 2012	M	56	Septic shock	AT, PBC, MV	Fever	NO	Discharged
T31	J.A.G.B.	December 2012	M	57	Pneumonia	AT, UC, PC	UTI	Fluconazole	Discharged
T32	P.A.M.	December 2012	M	97	Respiratory failure	AT, UC, PC	Fever	NO	Death

AT: antibiotic treatment, CC: central catheter, COPD: chronic obstructive pulmonary disease, MV: mechanical ventilation, NA: not available, PA: previous admission in the last year, PBC: permanent bladder catheter, PC: peripheral catheter, PS: previous surgery, TS: treatment with steroids, UC: urinary catheter

*Fungal isolates not available for molecular characterization

In vitro antifungal susceptibilities of *T. asahii* isolates to amphotericin B, 5-flucytosine, fluconazole, voriconazole, and caspofungin using the broth dilution method according to EUCAST guidelines [8], the Vitek 2 system, and Etest are summarized in Table 2. Four isolates did not grow enough on the test card of the Vitek system after the incubation time stipulated. The broth dilution method (BD) rendered significantly higher MIC_{50} and MIC_{90} values than Vitek 2 for all drugs except caspofungin, for which all the isolates were non-susceptible. However, the geometric mean (GM) of the MICs were in the range of ±1 dilution. The MIC values obtained by Etest for amphotericin B and 5-flucytosine were considerably higher than those by BD. However, the MICs for fluconazole and voriconazole were much lower with Etest than with the other methods. Comparing the GM of the MICs, the values obtained by Etest were similar for amphotericin B and much lower for the azoles. All isolates showed inexplicably high MICs with Etest for 5-flucytosine.

Itraconazole was assayed by both, broth dilution, and Etest, showing great differences in their MIC_{90} results which, as in the case of amphotericin B and 5-flucitosine, were significantly higher with Etest (Table 2).

Posaconazole was tested only by Etest. The MIC_{50}, MIC_{90}, and GM of the MICs and range were 0.25, 0.25, 0.18, and

Table 2 In vitro activities of antifungal agents comparing broth dilution (BD), Vitek 2, and Etest

	Amphotericin B			5-Flucytosine			Fluconazole			Voriconazole			Itraconazole			Caspofungin		
	BD	Vitek 2	Etest	BD	Vitek 2	Etest	BD	Vitek 2	Etest	BD	Vitek 2	Etest	BD	Vitek 2	Etest	BD	Vitek 2	Etest
MIC_{50}	1.0	0.5	2.0	8.0	4.0	≥32.0	8.0	2.0	1.5	0.25	0.1	0.06	0.5	NA	0.5	≥16.0	≥4.0	≥32.0
MIC_{90}	2.0	1.0	12.0	16.0	8.0	≥32.0	8.0	8.0	4.0	1.0	0.3	0.125	1.0	NA	6.0	≥16.0	≥4.0	≥32.0
GM	1.25	0.65	1.54	8.20	3.24	≥32.0	5.30	2.87	1.32	0.31	0.50	0.05	0.37	NA	0.72	≥16.0	≥4.0	≥32.0
Range	0.5–2	≤0.25–2	0.5–>32	2.0–32.0	≤1.0–8.0	≥32.0	2.0–32.0	2.0–16.0	0.1–64.0	0.1–1.0	≤0.12–1.0	0.008–0.75	0.12–1.0	NA	0.032–32.0	≥16.0	≥4.0	≥32.0

GM: geometric mean of the MICs, MIC: minimum inhibitory concentration in μg/mL, NA: not available

(0.008–1.5), respectively. Comparing the GM of the MICs obtained by Etest, the most active triazole was voriconazole, followed by posaconazole, itraconazole, and fluconazole.

All the *T. asahii* isolates studied belong to genotype I defined by Rodriguez-Tudela et al. [11].

The DiversiLab system generated rep-PCR DNA fingerprints for each of the 27 *T. asahii* isolates, and the similarity comparisons are shown as a dendrogram (Fig. 1). We found four clusters (A to D) for *T. asahii* isolates, each of which include both similar and indistinguishable strains; however, all isolates clustered with >90 % similarity. Four groups (A, B, C, D) were defined according to the criteria exposed in the Materials and methods section, including both indistinguishable and similar isolates. No relationship with epidemiological data, such as the date or clinical unit of hospitalization, was observed in the genotypic groups.

Discussion

Trichosporon spp. are medically important pathogens usually associated to indwelling medical devices in debilitated patients [3], with persistent infection or recurrence after treatment being frequent. Consistent with other reports [6, 11, 12], there were susceptible populations commonly associated with trichosporonosis in this study. All patients except for six were over 65 years old (median 81 years old) and all of them had urinary catheterization, were under antibiotic pressure, and had severe underlying diseases. In most cases, no antifungal treatment was administered to the patient, due, in some cases, to the lack of perception of the pathogenic action of this yeast.

No discrepancies were found between the Vitek 2 system and molecular methods used in order to identify the isolates to the species level. So, we consider that the Vitek 2 system could be a suitable method for routine use in many clinical microbiology laboratories.

Triazoles are among the most commonly studied drugs against *Trichosporon* [13, 14]; however, there are still uncertainties regarding the optimal drug to be chosen for treatment and there are few studies on the in vitro activity. The comparison of GM of the MIC values obtained by Etest for the triazoles showed that voriconazole was the most active agent, followed by posaconazole, itraconazole, and fluconazole, according to published results [14].

Considering BD as the reference method, all clinical isolates exhibited low MICs of voriconazole, agreeing with other studies [6, 7, 15]. In the present study, amphotericin B presented MIC values lower than those found in other works [7, 13] but similar to other studies [16–18]. Amphotericin B has long been a commonly used antifungal in the treatment of *Trichosporon* infections. However, although in vitro amphotericin B MICs could be low, the fungicidal effect of this drug may remain inadequate against some strains of

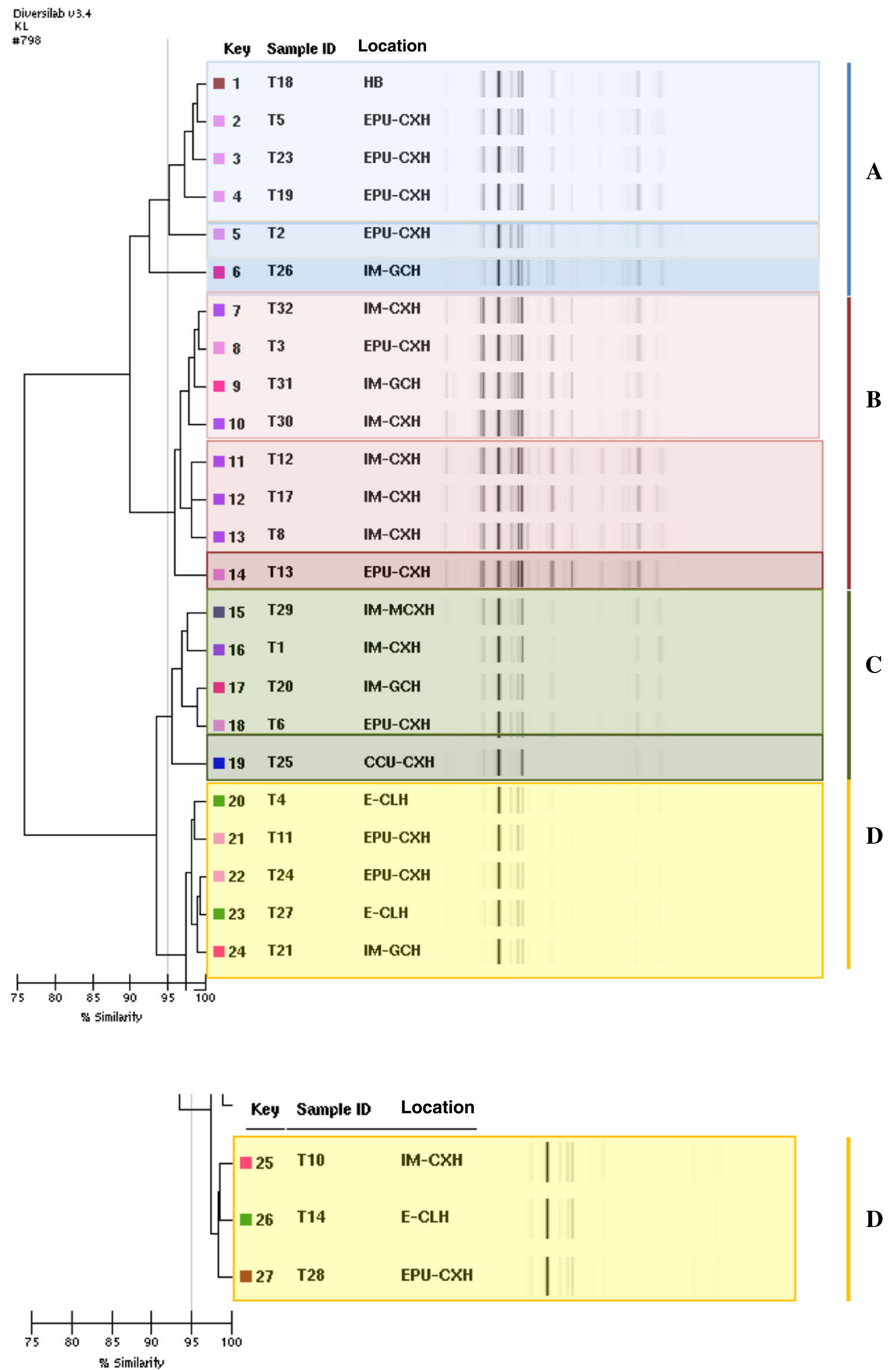

Fig. 1 Dendrogram obtained by repetitive-sequence-based polymerase chain reaction (rep-PCR) (DiversiLab) of *Trichosporon asahii* from the 27 studied patients using the Kullback–Leibler coefficient. *EPU-CXH* unit of elderly patients with serious underlying diseases in the CXH building; *IM-CXH* internal medicine unit in the CXH building; *IM-GCH* internal medicine unit in the GCH building; *E-CLH* emergency unit in the CLH building; *CCU-CXH* critical care unit in the CXH building; *HB* Hospital of Barbanza. Groups of fingerprint patterns defined: A, B, C, D

Trichosporon [19]. So, this drug should not be recommended as a treatment for trichosporonosis.

The activity of echinocandins is inherently limited against the genus *Trichosporon*, as was recognized by the three methods used for susceptibility testing.

Regarding the agreement among the three methods used for the susceptibility testing of amphotericin B, 5-flucytosine, fluconazole, voriconazole, and caspofungin, no differences higher than one dilution in the GM of the MICs were observed between BD and the Vitek 2 system according to other studies

[20], so, the Vitek 2 system could be used for the routine assay of the *T. asahii* susceptibilities in the clinical laboratory, considering that is a simpler method for everyday use. Etest is, also, a not too laborious method to be used in the routine laboratory. However, the results obtained with this method are discordant with the former, especially for 5-flucytosine, for which we consider that the results are aberrant. Lemes et al. [21] have also described large discrepancies between Etest and Clinical and Laboratory Standards Institute (CLSI) methodologies for 5-flucytosine and itraconazole.

The 27 clinical isolates studied belonged to genotype I comparing the IGS 1 sequences with those stored in the National Centre of Microbiology of Spain, as described by Rodriguez-Tudela et al. [11, 22]. However, this method does not distinguish between the monoclonal or polyclonal origin of the strains.

The DiversiLab system (bioMérieux, Marcy l'Etoile, France) is a rapid and technically simple method that uses rep-PCR for bacterial strain typing and has been used to characterize the genotypic relatedness among different fungi, such as *Candida*, *Aspergillus*, *Fusarium*, *Dermatophytes*, and *Zygomycetes* [22–27]. All isolates clustered with >90 % similarity, which has been described for the identification of other fungi [22, 23]. The DiversiLab system results may be analyzed using different coefficients: Pearson (PC) and Kullback–Leibler (KL). In PC analysis, both the band presence and the intensity are important. In KL analysis, the band presence is more important than the band intensity. Either way, the interpretation should be the same because it is based on the number of band differences. DiversiLab fingerprinting profiles demonstrated four different genotypic groups, but no specific epidemiological relationship is evidenced. We must keep in mind that the dendrogram provided by the DiversiLab software is not a tool for phylogenetic classification of microorganisms, but is an aid to identifying clusters and comparing the graphs within one cluster.

The University Hospital Complex of Santiago de Compostela consists of three buildings, called Clinical Hospital (CH), Conxo Hospital (CXH), and Gil Casares Hospital (GCH), respectively. So, in relation to their location in the hospital, all patients except one had been admitted into the internal medicine unit of the CX building at the time of the *T. asahii* isolation or in the previous months. The exception was the youngest patient, who was in the critical care unit and had a disseminated infection by *T. asahii*. The building and the urinary catheterization were the only epidemiological data in common with the rest of the patients.

Because the fingerprints patterns were different among the established groups, we cannot conclude that we are dealing with an outbreak, and it could be that *T. asahii* was an emergent pathogen in elderly and seriously ill patients.

In summary, *T. asahii* is, likely, an emergent pathogen in elderly patients with urinary drainage devices that can be adequately treated with triazoles, with voriconazole being the most active. On the other hand, the Diversilab system for clonal strain typing may be a useful tool for fast and accurate management of nosocomial outbreaks.

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Conflict of interest We have no conflicts of interest to declare.

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