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RESEARCH ARTICLE

Genotyping, antifungal susceptibility testing, and biofilm formation of *Trichosporon* spp. isolated from urine samples in a University Hospital in Bangkok, Thailand

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ABSTRACT

The basidiomycetes yeast *Trichosporon* is widespread in the natural environment, but can cause disease, mainly in immunocompromised patients. However, there have been only few studies about this infection in Thailand. In this study, we characterized 53 *Trichosporon* spp. isolated from urine samples from patients admitted to a single hospital in Bangkok, Thailand over a one-year period from 2019 to 2020. The strains were identified using colony morphology, microscopy, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, and nucleotide sequence analysis of intergenic spacer 1 (IGS1). Fifty-one isolates were *Trichosporon asahii*, and the remaining isolates were *Trichosporon inkin* and other *Trichosporon* species. Three genotypes of IGS1—1, 3, and 7 were observed among *T. asahii*. The sensitivity of the yeasts to the antifungal drugs amphotericin B, fluconazole, and voriconazole ranged from 0.25 to >16 μg ml^{-1}, 0.5–8 μg ml^{-1}, and 0.01–0.25 μg ml^{-1}, respectively. We investigated biofilm formation by the isolates, and no biofilm production was found in one isolate, low biofilm production in forty-four isolates, and medium biofilm production in six isolates. *T. inkin* produced biofilms at low levels, and *Trichosporon* spp. produced biofilms at medium levels. This research increases our understanding of the molecular epidemiology of *Trichosporon* spp. isolated from one university hospital in Bangkok, Thailand, and reveals their genetic diversity, antifungal susceptibility profiles, and capacity for *in vitro* biofilm production.

KEYWORDS

Trichosporon species, genotyping, susceptibility testing, biofilm

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INTRODUCTION

Trichosporon spp. are yeast-like fungi that are normally found in environmental niches, such as soil, seawater, rivers, lake, decayed wood, leaf mold, and animals [1–3]. This genus grows

well on Sabouraud dextrose agar (SDA) and forms colonies, which are white to cream-colored, raised, waxy, contain radial furrows, and cerebriform or irregular folds. Microscopic observation of *Trichosporon* spp. reveals blastoconidia, hyaline septate hyphae, and arthroconidia.

Trichosporon spp. cause infections ranging from superficial fungal infections, such as white piedra and onychomycosis, to fatal disseminated infection. Trichosporonosis is an invasive infection caused by *Trichosporon* spp., particularly in patients with hematologic malignancies [1]. Breakthrough *Trichosporon* spp. infections have been reported in patients exposed to antifungal drugs, for either prophylaxis or therapy [4]. The most relevant clinical species are *T. asahii, T. asteroides, T. inkin,* and *T. ovoides* [5].

There are several virulence factors in *Trichosporon* spp. that facilitate the development of disease, including biofilm formation and protease and lipase production [1]. Previous studies have demonstrated the ability of *Trichosporon* spp., to form biofilms on biomaterial-representative polystyrene surfaces, and showed their high resistance to antifungal drugs, such as triazole and amphotericin B [6, 7]. Therefore, the capacity of *Trichosporon* spp. to form biofilms on medical devices results in medical device-related infection.

Molecular techniques are widely used to identify *Trichosporon* spp., especially polymerase chain reaction (PCR)-based methods. PCR and DNA sequencing of the internal transcribed spacer (ITS) region, the D1/D2 region of the 28S rDNA, and the intergenic spacer 1 (IGS1) region are the most frequently used techniques for the identification of species [5, 8, 9]. In addition, IGS1 is also used for the genotyping of *T. asahii* [10]. Moreover, a previous study by Nobrega de Almeida et al., identified other loci in *T. asahii* with high haplotypic diversity, including topoisomerase 1, phosphate carrier protein, beta-1-tubulin, and copper-exporting ATPase [11].

Thus, the aim of this investigation was to identify the genotypic distribution of clinical isolates of *Trichosporon* spp. from the University Hospital, Bangkok, Thailand, using IGS1 for sequence-based identification, along with their antifungal susceptibility profiles and biofilm formation capability.

MATERIALS AND METHODS

Study population and identification

Fifty-three isolates of *Trichosporon* spp. from urine sample cultures ($\geq 10^4$ colony-forming unit per milliliter) [12] were collected from the Microbiology Laboratory, Department of Central Laboratory and Blood Bank, Faculty of Medicine, Vajira Hospital between October 2019 and September 2020. The yeasts were first identified to the species level using a MALDI BioTyper (Bruker, Daltonik GmbH, Bremen, Germany) and stored in distilled water at 4 °C.

Genomic DNA extraction, molecular identification, and genotyping analysis

Yeasts were cultured on SDA and maintained at 35 °C for 48 h. Genomic DNA was extracted and modified according to a previously published method [13]. In brief, a loop full of *Trichosporon* colonies was transferred into a 1.5 ml micro centrifuge tube and frozen at −20 °C for 1 h. Lysis buffer (500 µL) was added (0.5 g sodium dodecyl sulfate, 1.4 g NaCl, 0.73 g EDTA, 20 mL 1 M Tris–HCl per 100 mL lysis buffer, and 5 µL of 2-mercaptoethanol) and the suspension was vortexed and incubated at 65 °C for 1 h. The lysate was extracted using 500 µL phenol–chloroform–isoamyl alcohol (25:24:1, v:v:v) (SIGMA® Life Science, St. Louis, Missouri, USA) and mixed by pipetting. The tubes were centrifuged for 5 min at 12,500 rpm at 4 °C. The aqueous phase was transferred to a new tube, and DNA was precipitated with 500 µL of isopropanol and incubated for 1 h at 4 °C. To pellet the DNA, the solution was centrifuged for 5 min at 12,500 rpm at 4 °C. The DNA pellet was washed with 70% ethanol, centrifuged for 5 min at 12,500 rpm, and air-dried. The DNA was resuspended in 100 µL of sterile deionized water and stored at −20 °C. The genus *Trichosporon* was confirm using PCR with the specific primer pair TRF (5′-AGAGGCCTACCATGGTATCA-3′) and TRR (5′-TAAGACCCAATAGAGCCCTA-3′) [14, 15]. Each PCR reaction mixture (50 µl final volume) contained 0.5 µM of each primer, exTEN 2X PCR Master Mix with loading dye (Axil Scientific Pte Ltd, Singapore), nuclease-free water, and genomic DNA. PCR amplification was performed using a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) as follows: initial step at 95 °C for 5 min, followed by 35 cycles of amplification at 95 °C for 1 min, annealing at 55 °C for 45 s, extension at 72 °C for 45 s, and a final extension at 72 °C for 5 min. The negative control was done using a DNA template of *Candida albicans* ATCC 90028.

Then, the isolates were identified to the species level and genotype using PCR and sequence analysis of IGS1. PCR was performed using specific primer pairs with the primers 26SF (5-ATCCTTTGCAGACGACTTGA-3) and 5SR (5-AGCTTGACTTCGCAGATCGG-3) [14, 16]. Each PCR reaction mixture (50 µl final volume) contained 0.5 µM of each primer, exTEN 2X PCR Master Mix with loading dye (Axil Scientific Pte Ltd, Singapore), nuclease-free water, and genomic DNA. The PCR amplification protocol was modified slightly from that published by Sugita et al. [14, 16] by using a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) as follows: initial step at 95 °C for 5 min, followed by 35 cycles of amplification at 95 °C for 1 min, annealing at 57 °C for 45 s, extension at 72 °C for 45 s, and a final extension at 72 °C for 5 min.

Five microliters of PCR products (genus specific and IGS1) were electrophoresed on a 1.5% agarose gel in 1X TBE buffer containing FluoroSafe DNA stain (Axil Scientific Pte Ltd, Singapore), and photographed using a Gel Doc XR + system (Bio-Rad, Hercules, CA, USA). For IGS1, each PCR product was purified and bidirectionally sequenced using gene-specific forward and reverse primers at 1st BASE DNA Sequencing (Apical Scientific Sdn Bhd, Malaysia) by BNK Bioscience Limited, Nonthaburi, Thailand. The sequence files were analyzed using BioEdit software (https://www.mbio.ncsu.edu/bioedit/bioedit.html) and compared with sequences in GenBank [17] using BLASTn (https://blast.ncbi.

nlm.nih.gov/Blast.cgi) for species identification and genotyping analysis.

To assess the evolutionary relationships among the strains studied, the sequences were aligned and trimmed to the correct length from the start to the end of the IGS1 sequences. The IGS1 sequences of *Trichosporon* spp. were download from the GenBank (*T. asahii* strain FHD01; MK737687.1, *T. asahii* strain TN74U09; KX017586.1, *T. asahii* strain CBS 8972; MK737692.1, *T. asahii* strain FNA01; MK737679.1, *T. asahii* strain CBS 5599; MK737694.1, *T. asahii* FNA02; MK737680.1, *T. inkin* strain HCFMUSP-DLC03; KY421049.1, *T. ovoides* strain CBS 7556; EU934805.1, *T. coremiiforme* strain L03-001; KM488273.1, *T. asteroides* strain L9737; KY312716.1, and *T. faecale* strain CBS 4828; KM488293.1). IGS1 sequences of *Trichosporon mycotoxinivorans* (recently reclassified as *Apiotrichum mycotoxinivorans*) (*T. mycotoxinivorans* strain HB 1175; AJ601390.1) and *Trichosporon debeurmannianum* (recently reclassified as *Cutaneotrichosporon debeurmannianum*) (*C. debeurmannianum* strain HCFMUSP-DLC101; KY657455.1) were used as outgroups. The evolutionary history was inferred using the maximum likelihood method. The best models of evolution for the data set were selected using the Bayesian Information Criterion (BIC) of MEGA 11 [18]. The model with the lowest BIC score was chosen to construct as the basis for the construction of maximum likelihood phylogenetic trees. The percentage of trees in which the associated taxa clustered together is shown next to each branch. The initial tree for the heuristic search was automatically obtained by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and then selected the topology associated with a superior log likelihood value was selected. The tree is drawn to scale, with branch lengths representing the number of substitutions per site. A bootstrap analysis was conducted using 500 replicates, and bootstrap values ≥50% are shown above the branches.

Antifungal susceptibility testing

We tested the susceptibility of the yeast samples to amphotericin B; AMB (0.03–16 μg ml^{-1}), fluconazole; FLU (0.125–64 ug/ml), and voriconazole; VOR (0.01–8 ug/ml) (Sigma-Aldrich, St. Louis, MO, USA) using the CLSI broth microdilution method according to the document M27-A3 [19, 20]. Stock solutions of antifungal drugs were prepared in dimethyl sulfoxide (DMSO). Each drug was diluted in Roswell Park Memorial Institute (RPMI) 1640, without bicarbonate, with phenol red, buffer with 0.165 mmol L^{-1} of M-morpholinepropanesulfonic pH 7.0 (Gibco, Grand Island, NY, USA) in twice the final concentration in round bottom 96-well plates with lids (costar®, Corning Incorporated, Kennebunk, ME, USA). One hundred microliters of each antifungal drug was placed into each well along with the 2X inoculum suspension. Serial dilutions were then performed to achieve the concentrations listed above.

Before testing, yeasts were subcultured on SDA at 35 °C for 48 h. The inoculum suspensions of fungal isolates were prepared in 0.9% saline solution and adjusted to a turbidity of 0.5 McFarland standard in a concentration of 1–5 × 10^6 cells/ml [21]. The final concentration of the inoculums was 1–5 × 10^3 cell/ml. All plates were incubated at 35 °C for 48 h. The minimum inhibitory concentration (MIC) of amphotericin B was defined as the lowest concentration of the drug that substantially inhibited 100% growth of the organism, and for fluconazole and voriconazole as that which inhibited 50% of growth, as detected visually. For the conventional microdilution procedure, the growth in each well was compared with the growth of the control with the aid of a reading mirror. The MICs of 50% and 90% of the total population were defined as the MIC50 and MIC90, respectively. Drug free and yeast controls were included.

Quality control organisms were included in all experiments, and were represented by the *C. albicans* ATCC 90028, *Candida krusei* ATCC 6258, and *Candida parapsilosis* ATCC 22019 strains. An isolate was considered to have a high MIC if the isolates high above epidemiological cut-off values (ECV) after 48 h of incubation, as proposed by Francisco et al. [22]. The ECVs of AMB, FLU, and VOR were 4 μg ml^{-1}, 8 μg ml^{-1}, and 0.25 μg ml^{-1}, respectively.

Biofilm formation under static conditions

A biofilm formation assay was adapted from previously described assays [23–26]. In brief, the *Trichosporon* spp. were cultured on SDA at 35 °C for 48 h, and further subcultured under agitation in Roswell Park Memorial Institute 1640-plus-3-(*N*-morpholino) propanesulfonic acid medium, pH 7.0 (RPMI 1640) overnight at 37 °C. The cells were collected using centrifugation and washed with sterile phosphate-buffered saline (PBS). Cells were resuspended in RPMI 1640 and the inoculum adjusted to 0.1 O.D. at an absorbance of 600 nm [A600] using an OD600 DiluPhotometer™ (Implen GmbH, München, Germany). Then, 100 μL of adjusted cell suspension was inoculated into each well of a flat-bottom 96-well plate with lid (costar®, Corning Incorporated, Kennebunk, ME, USA), using eight wells per isolate. The plates were incubated without shaking at 37 °C for 1 h, the supernatants were removed, and the cells were washed with PBS. Next, fresh RPMI medium was added, followed by incubation without shaking at 37 °C for 24 h. Subsequently, the supernatant and planktonic cells were removed after a further 24 h of incubation, the wells were washed with PBS, and then fresh RPMI medium was added. The experiment was done in duplicate, and biofilm measurements are expressed as the means of 16 readings (wells) per isolate ± SD. Semi-quantitative evaluation of biofilm formation was performed using 0.4% crystal violet (CV) staining. After the supernatant was removed and the cells washed with PBS, the plates were dried for 45 min at room temperature. Biofilms were stained with 110 μl 0.4% CV solution for 45 min. The CV solution was then removed and the wells washed three times with 200 μL of sterile water. To destain the biofilms, 200 μl 95% ethanol was added, and the plate was incubated for 45 min. One hundred microliters of the solution were transferred to another microplate, and the

absorbance was read at 570 nm using a Tecan's Sunrise absorbance microplate reader (Tecan Austria GmbH, Grödig, Austria). The production of biofilm was quantified using CV and categorized following the method described by Iturrieta-González et al. Low biofilm production was A570 < 1, medium biofilm production was A570 ≥ 1.01 to 2.499, and high biofilm production was A570 ≥ 2.5 [24].

Data availability

The nucleotide sequences were deposited in GenBank under accession numbers ON051546 to ON051598 for IGS1 sequences.

RESULTS

Strain identification and genotyping analysis

In the one-year period of our study (October 2019 to September 2020), we first collected *Trichosporon* spp. from all clinical specimens in our hospital, but we could isolate the strains only from urine samples. A total of 53 isolates of *Trichosporon* spp. were collected, of which 52 were identified as *T. asahii* and one as *T. inkin* using a MALDI-TOF biotyper in the Microbiology Laboratory of our hospital (Table 1). All isolates grew well on SDA after incubation at 35 °C for 48 h. The colonies were white to cream in color, wrinkled, with cerebriform or irregular fold characteristics.

We used PCR with the specific primers TRR and TRF to confirm that the isolates belonged to the genus *Trichosporon*. The PCR products of all isolates were approximately 170 base pairs (bp), confirming that all isolates tested were *Trichosporon* spp. We further identified the studied strains to the species level using PCR and sequence-based identification of the IGS1 gene. Out of the 53 isolates, 51 isolates were identified as *T. asahii*, one was identified as *T. inkin* (VJR-TRCINK008), and one isolate was not identified at the species level, and was thus designated *Trichosporon* spp (VJR-TRCSPP006). The species closest to the unidentified species was *T. asahii*. The IGS1 sequence similarity between the unknown isolate and *T. asahii* strain CBS 5599 (GenBank accession No. MK737694.1) was 90.81%, according to a BLAST search undertaken on January 10, 2022.

We analyzed the genotypes of the 51 *T. asahii* isolates using the IGS1 gene, and found three different genotypes corresponding to previously published genotypes [26–32] as summarized in Table 1. These genotypes were deposited in GenBank; 10 isolates were Genotype 1, eight were Genotype 3, and 33 were Genotype 7 (Tables 1 and 2). Evolutionary analyses of IGS1 sequences were conducted using MEGA11 [18]. The K2: Kimura 2-parameter model [33] and the non-uniformity of evolutionary rates among sites may be modeled by assuming that a certain fraction of sites are evolutionarily invariable (+I), a value that was chosen according the best model of evolution analyses. The BIC score was 10208.362087046. Then, a phylogenetic tree was constructed using maximum likelihood analysis based on the K2+I model with 500 replications of bootstrap analysis. The tree with the highest log likelihood, 4635.59 was selected. The rate variation model allows for some sites to be evolutionarily invariable ([+I], 23.66% sites). Sixty-six

Table 1. Genus and species identification of *Trichosporon* spp.

Strain	MALDI-TOF identification	IGS1	IGS1 (Genotype)
VJR-TRCASH001	*T. asahii*	*T. asahii*	7
VJR-TRCASH002	*T. asahii*	*T. asahii*	3
VJR-TRCASH003	*T. asahii*	*T. asahii*	7
VJR-TRCASH004	*T. asahii*	*T. asahii*	3
VJR-TRCASH005	*T. asahii*	*T. asahii*	7
VJR-TRCSPP006	*T. asahii*	*Trichosporon* spp.	-
VJR-TRCASH007	*T. asahii*	*T. asahii*	7
VJR-TRCINK008	*T. inkin*	*T. inkin*	-
VJR-TRCASH009	*T. asahii*	*T. asahii*	7
VJR-TRCASH010	*T. asahii*	*T. asahii*	7
VJR-TRCASH011	*T. asahii*	*T. asahii*	7
VJR-TRCASH012	*T. asahii*	*T. asahii*	7
VJR-TRCASH013	*T. asahii*	*T. asahii*	7
VJR-TRCASH014	*T. asahii*	*T. asahii*	7
VJR-TRCASH015	*T. asahii*	*T. asahii*	7
VJR-TRCASH016	*T. asahii*	*T. asahii*	7
VJR-TRCASH017	*T. asahii*	*T. asahii*	7
VJR-TRCASH018	*T. asahii*	*T. asahii*	7
VJR-TRCASH019	*T. asahii*	*T. asahii*	7
VJR-TRCASH020	*T. asahii*	*T. asahii*	3
VJR-TRCASH021	*T. asahii*	*T. asahii*	7
VJR-TRCASH022	*T. asahii*	*T. asahii*	7
VJR-TRCASH023	*T. asahii*	*T. asahii*	7
VJR-TRCASH024	*T. asahii*	*T. asahii*	3
VJR-TRCASH025	*T. asahii*	*T. asahii*	3
VJR-TRCASH026	*T. asahii*	*T. asahii*	1
VJR-TRCASH027	*T. asahii*	*T. asahii*	7
VJR-TRCASH028	*T. asahii*	*T. asahii*	7
VJR-TRCASH029	*T. asahii*	*T. asahii*	1
VJR-TRCASH030	*T. asahii*	*T. asahii*	7
VJR-TRCASH031	*T. asahii*	*T. asahii*	7
VJR-TRCASH032	*T. asahii*	*T. asahii*	3
VJR-TRCASH033	*T. asahii*	*T. asahii*	7
VJR-TRCASH034	*T. asahii*	*T. asahii*	7
VJR-TRCASH035	*T. asahii*	*T. asahii*	7
VJR-TRCASH036	*T. asahii*	*T. asahii*	7
VJR-TRCASH037	*T. asahii*	*T. asahii*	1
VJR-TRCASH038	*T. asahii*	*T. asahii*	7
VJR-TRCASH039	*T. asahii*	*T. asahii*	1
VJR-TRCASH040	*T. asahii*	*T. asahii*	7
VJR-TRCASH041	*T. asahii*	*T. asahii*	1
VJR-TRCASH042	*T. asahii*	*T. asahii*	7
VJR-TRCASH043	*T. asahii*	*T. asahii*	1
VJR-TRCASH044	*T. asahii*	*T. asahii*	7
VJR-TRCASH045	*T. asahii*	*T. asahii*	7
VJR-TRCASH046	*T. asahii*	*T. asahii*	1
VJR-TRCASH047	*T. asahii*	*T. asahii*	7
VJR-TRCASH048	*T. asahii*	*T. asahii*	1
VJR-TRCASH049	*T. asahii*	*T. asahii*	7
VJR-TRCASH050	*T. asahii*	*T. asahii*	1
VJR-TRCASH051	*T. asahii*	*T. asahii*	3
VJR-TRCASH052	*T. asahii*	*T. asahii*	1
VJR-TRCASH053	*T. asahii*	*T. asahii*	3

Table 2. Distribution of *T. asahii* genotypes in Thailand

Year of Isolated	No. of *T. asahii*	G1	G2	G3	G5	G7	References
1997–2007	101	45 (44.5%)	2 (2.0%)	35 (34.7%)	1 (1.0%)	18 (17.8%)	Mekha et al. [34]
2019–2020	51	10 (19.6%)	-	8 (15.7%)	-	33 (64.7%)	Present study

nucleotide sequences were included in the tree (Fig. 1). The codon positions included were $1^{st} + 2^{nd} + 3^{rd}$ + Noncoding. There were 663 positions in the final dataset. *T. asahii* was strongly clustered into Genotype 1 (bootstrap value = 98%), Genotype 3 (bootstrap value = 100%), and Genotype 7 (bootstrap value = 95%). The isolates of VJR-TRCINK008 clustered with the references sequence of *T. inkin* (bootstrap value = 100%). One isolate, VJR-TRCSPP006, was not identified to the species level, and stood alone in the tree (bootstrap value = 100%) (Fig. 1). This isolate was identified as *T. asahii* using MALDI-TOF.

Antifungal susceptibility testing

Among the 51 *T. asahii* isolates, the MIC value of AMB, FLU, and VOR ranged from 0.25 to >16 μg ml^{-1}, 0.5–8 μg ml^{-1}, and 0.01–0.25 μg ml^{-1}, respectively (Appendix Table A1 and Table 3). The MIC 50 and MIC 90 values are shown in Table 3. Seven isolates showed high MIC for AMB (4 ug/ml for six isolates and >16 μg ml^{-1} for one isolate). The MIC of FLU from two isolates was 8 μg ml^{-1} and the MIC of VOR from one isolate was 0.25 μg ml^{-1}. The VJR-TRCASH037 showed high MIC for both FLU (8 μg ml^{-1}) and VOR (0.25 μg ml^{-1}). The MIC values of AMB, FLU, and VOR of VJR-TRCINK008 (*T. inkin*) were 0.5 μg ml^{-1}, 0.5 μg ml^{-1}, and 0.03 μg ml^{-1}, respectively. The MIC values of AMB, FLU, and VOR of VJR-TRCSPP006 (*Trichosporon* spp.) were 2 μg ml^{-1}, 1 μg ml^{-1}, and 0.06 μg ml^{-1}, respectively.

Biofilm production

The biofilm formation capability of the 51 isolates of *T. asahii* was determined by measuring the absorbance after CV staining of biofilms formed in flat-bottomed microtiter plates. The biofilm formation capacity was classified according to the scheme of Iturrieta-González et al. [24]. We found a non-biofilm forming isolate (VJR-TRCASH028), 44 low biofilm forming isolates (A570 < 1), and six medium biofilm forming isolates (A570 ≥ 1.01–2.499), as shown in Fig. 2. *T. inkin* produced low levels of biofilm, and *Trichosporon* spp. produced medium levels (Appendix Table A1).

DISCUSSION

Trichosporon species are basidiomycetous yeasts which are capable of forming true hyphae. They are widely distributed in the environment, and are present in the human flora. However, *Trichosporon* can be pathogenic, causing human infections such as superficial white piedra, invasive trichosporonosis, and fungemia. Risk factors include an immunocompromised host, hematologic malignancies, AIDS, corticosteroid treatment, long length of hospital/ICU stay, and catheterization [35]. *T. asahii* is the species most commonly identified as a pathogen [35]. There have been several reported cases of *Trichosporon* infection in COVID-19 patients, including brain abscess caused by *Trichosporon dohaense* [36], and nosocomial pneumonia [37], urinary tract infection [38], and fungemia [11, 35] caused by *T. asahii*. In Thailand, there have been some reports of *Trichosporon* spp. isolated from clinical specimens. Anunnatsiri et al. investigated the incidence of fungemia in non-HIV-infected patients, and found that *Trichosporon* accounted for 6.1% of the cases between 1999 and 2003 [39]. In 2014, Issarachaikul et al. described five cases report of catheter-related bloodstream infections from *Trichosporon* spp. in patients with risk factors and the prolonged presence of a central venous catheter [40]. In this study, our *Trichosporon* spp. were collected from urine samples.

In a review article, Takashima et al. [41], updated the genus *Trichosporon* to comprise the following species: *T. inkin*, *T. ovoides*, *T. caseosum*, *T. lactis*, *T. dohaense*, *T. coremiiforme*, *T. asahii*, *T. faecale*, *T. insectorum*, *T. asteroids*, *T. japonicum*, and *T aquatile*. The other species previously classified in the genus *Trichosporon* were reclassified and assigned into four other genera: *Apiotrichum* (such as *A. mycotoxinivorans*), *Cutaneotrichosporon* (such as *C. cutaneum* and *C. mucoides*), *Effuseotrichosporon* (*E. vanderwaltii*), and *Haglerozyma* (*H. chiarellii*) as proposed by Liu et al. [42, 43]. It is difficult to distinguish closely related species in the genus *Trichosporon* using PCR and sequence-based identification of the ITS region, compared with the intergenic spacer (IGS) [41, 44], particularly among *T. asahii*, *T. coremiiforme*, and *T. faecale* [41, 43]. Thus, we used a region of IGS1 to confirm the identification, genetic diversity and relationships of our isolates. We confirmed that our strains of *Trichosporon* were 51 isolates of *T. asahii*, one isolate of *T. inkin*, and one isolate of *Trichosporon* spp. Based on the IGS1 sequence diversity of *T. asahii*, Sugita et al. were the first team to describe five genotypes (Genotype 1 to Genotype 5) from 43 isolates of *T. asahii*, the major cause of deep-seated trichosporonosis, from Japan and the USA [15]. Genotypes 6 to 15 were described following [27–32]. There has only been one study into the identification of *T. asahii* genotypes in Thailand until now (January 10, 2022); a report by Mekha et al. in 2010 [34] (Table 2.). These researchers analyzed the genotypes of the IGS1 gene of 101 *T. asahii* isolates obtained from Thai patients collected between 1997 and 2007, and five genotypes were identified (Genotypes 1, 2, 3, 5, and 7) [34]. Our strains were Genotype 7 (64.7%), 1 (21.6%), and 3 (14.7%). We found only three out of five genotypes identified in the isolates studied 10–20 years ago in Thailand (Mekha et al. [34]), because our isolates were collected from only one hospital and only over a

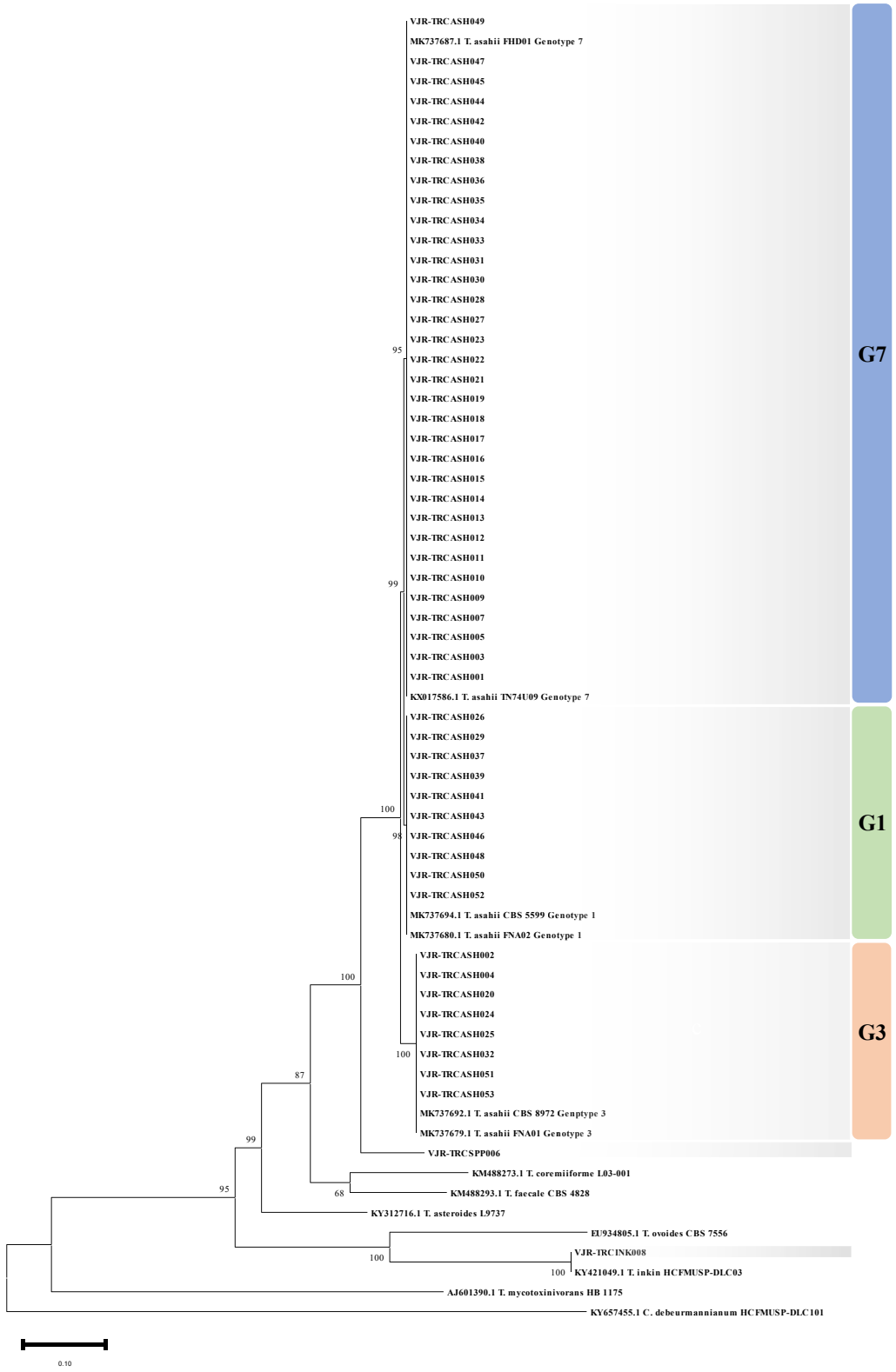

Fig. 1. Phylogenetic tree of IGS1 sequences. The percentage of trees in which the associated taxa clustered together is shown next to each branch. The initial tree for the heuristic search was obtained automatically by applying the Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with the highest log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site

Table 3. Susceptibility of the studied strains

IGS1 Genotype of *T. asahii* (N)	Antifungal agents	MIC range μg ml^{-1}	MIC 50 μg ml^{-1}	MIC 90 μg ml^{-1}
1 (10)	Amphotericin B	0.25–1	0.5	1
	Fluconazole	1–8	1	2
	Voriconazole	0.01–0.25	0.03	0.06
3 (8)	Amphotericin B	0.5–4	1	2
	Fluconazole	0.5–2	1	2
	Voriconazole	0.03–0.06	0.03	0.06
7 (33)	Amphotericin B	0.5– >16	1	4
	Fluconazole	1–8	2	4
	Voriconazole	0.03–0.12	0.12	0.06
All (51)	Amphotericin B	0.25 – >16	1	4
	Fluconazole	0.5–8	2	2
	Voriconazole	0.01–0.25	0.06	0.12

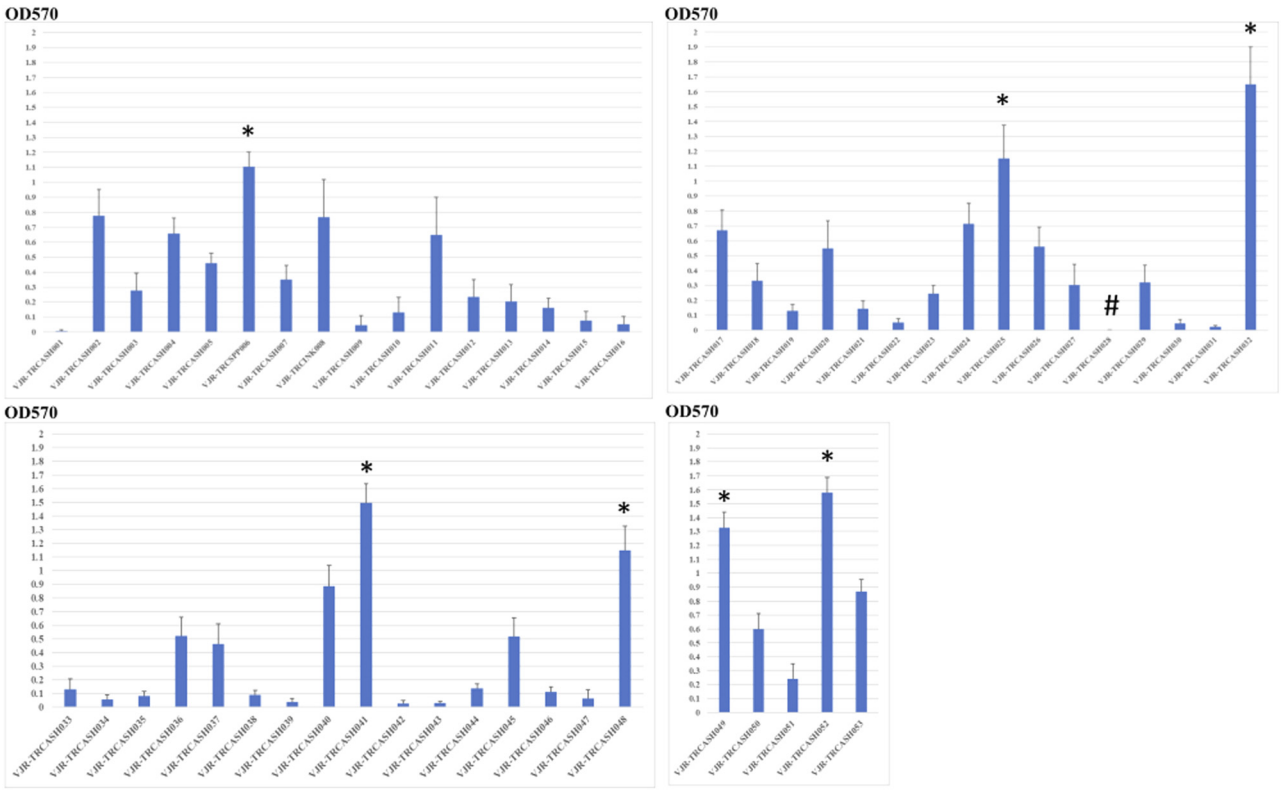

Fig. 2. Biofilm formation ability of the strains in this study. The bars represent the mean + standard deviation (SD) of the measured absorbance (OD570). * = medium biofilm forming isolates (A570 ≥ 1.01–2.499), # = non-biofilm forming isolate (VJR-TRCASH028), other strains = low biofilm forming isolates (A570 < 1)

short period, one year. Our predominant genotype was Genotype 7, whereas Mekha et al. found Genotypes 1 and 3 predominated in Thailand at that time (Table 2) [34]. The prevalence of Genotype 7 in this study was different from another report about *T. asahii* genotypes, in which Genotype 1 was observed to be the most common genotype in other countries: Brazil [26, 45], China [46], India [47], France [48], Mexico [49], Taiwan [50], Tunisia [14], and Turkey [51]. We could not identify strain VJR-TRCASH006 to the species level, but further characterization of this strain may lead to better identification, possibly as a new species.

When we conducted antifungal susceptibility testing, clinical breakpoints with which to interpret the MICs of antifungal agents for *Trichosporon* spp. were not available [52]. Therefore, we used the ECVs proposed by Francisco et al. [22]. In this study, we analyzed the current susceptibility profile of *Trichosporon* spp. isolated from 2019 to 2020 in one university hospital. We observed that the strains of *T. asahii* had higher MICs than the ECVs of AMB, FLU, and VOR in seven, two, and one isolates, respectively. Therefore, FLU and VOR were more potent antifungal agents *in vitro* against *T. asahii* in this population, a finding which was consistent with other studies in which a high number of

isolates showed a high MIC for AMB [46, 49, 53]. *T. inkin* and *Trichosporon* spp. were susceptible to all antifungal agents tested *in vitro*.

Fungal biofilm formation by clinical strains of *Trichosporon* spp. was investigated in several previous studies [12, 24, 26, 51, 54, 55]. The biofilms were established on medical devices such as catheters, and then caused invasive fungal infections and antifungal drug resistance [26]. In our work, most of the *T. asahii* stains (44 out of 51) and *T. inkin* were considered to be low biofilm producers, six isolates of *T. asahii* and *Trichosporon* spp. were classified as medium biofilm producers, and one isolate (VJR-TRCASH028) could not produce biofilm on a surface, according to the classification of Iturrieta-González et al. [24]. The ability of *Trichosporon* spp. isolated from urine to produce biofilms on polystyrene surfaces was previously investigated [12, 29]. Wei et al., observed that all of 23 clinical isolates *T. asahii* from UTI patients could form biofilms and a remarkably preformed-biofilm of *T. asahii* cell were resistant to all antifungal agents tested [12]. Almeida et al., observed 16 out of 68 *T. asahii* isolates from urinary catheterization could form biofilms [29]. This ubiquity of biofilm formation has important clinical implications, and the biofilms may be more resistant to antifungal agents than planktonic cells [12, 56].

There are several limitations to our present study. The isolates analyzed were collected from just one hospital; the collection period was short, at one year; and the samples were isolated only from urine samples.

In conclusion, we investigated a population of *Trichosporon* spp. from clinical specimens collected at a university hospital. Analysis based on the sequences of the IGS1 gene was used to study in the genetic diversity and distribution of *Trichosporon* spp. We further report the *in vitro* antifungal susceptibility profiles and *in vitro* biofilm formation capabilities of Thai isolates.

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Competing interests: The authors declare that they have no conflict of interest.

Ethics approval: The Ethical Review Board of the Faculty of Medicine, Vajira Hospital, Navamindradhiraj approved the study (COA130/2562).

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Appendix

Table A1. Susceptibility testing profile and biofilm production

Strains	Amphotericin B µg/ml	Fluconazole µg/ml	Voriconazole µg/ml	Biofilm production	
				A570 (mean)	SD
VJR-TRCASH001	4	2	0.12	0.0073	0.0070
VJR-TRCASH002	1	0.5	0.03	0.7778	0.1727
VJR-TRCASH003	1	2	0.06	0.2785	0.1143
VJR-TRCASH004	1	2	0.06	0.6582	0.1038
VJR-TRCASH005	0.5	1	0.06	0.4597	0.0678
VJR-TRCSPP006	2	1	0.06	1.1029	0.0980
VJR-TRCASH007	2	2	0.12	0.3516	0.0932
VJR-TRCINK008	0.5	0.5	0.03	0.7671	0.2504
VJR-TRCASH009	4	4	0.06	0.0451	0.0639
VJR-TRCASH010	4	4	0.06	0.1296	0.1004
VJR-TRCASH011	4	2	0.06	0.6479	0.2511
VJR-TRCASH012	2	2	0.06	0.2359	0.1142
VJR-TRCASH013	1	2	0.06	0.2033	0.1129
VJR-TRCASH014	1	2	0.06	0.1600	0.0643
VJR-TRCASH015	1	2	0.06	0.0749	0.0608
VJR-TRCASH016	1	2	0.06	0.0507	0.0535
VJR-TRCASH017	0.5	2	0.06	0.6713	0.1360
VJR-TRCASH018	0.5	8	0.12	0.3307	0.1165
VJR-TRCASH019	0.5	2	0.06	0.1283	0.0454
VJR-TRCASH020	0.5	1	0.03	0.5482	0.1875
VJR-TRCASH021	1	2	0.06	0.1447	0.0514
VJR-TRCASH022	1	2	0.06	0.0515	0.0243
VJR-TRCASH023	0.5	2	0.06	0.2450	0.0544
VJR-TRCASH024	2	1	0.06	0.7156	0.1364
VJR-TRCASH025	2	0.5	0.03	1.1516	0.2231
VJR-TRCASH026	1	1	0.03	0.5599	0.1315
VJR-TRCASH027	0.5	2	0.12	0.3036	0.1379
VJR-TRCASH028	1	2	0.12	-0.0049	0.0078
VJR-TRCASH029	0.5	1	0.03	0.3200	0.1166
VJR-TRCASH030	2	2	0.06	0.0449	0.0265
VJR-TRCASH031	2	2	0.06	0.0206	0.0097

(continued)

Table A1. Continued

Strains	Amphotericin B µg/ml	Fluconazole µg/ml	Voriconazole µg/ml	Biofilm production	
				A570 (mean)	SD
VJR-TRCASH032	4	2	0.06	1.6499	0.2501
VJR-TRCASH033	1	2	0.12	0.1279	0.0797
VJR-TRCASH034	1	2	0.06	0.0522	0.0362
VJR-TRCASH035	1	2	0.06	0.0813	0.0318
VJR-TRCASH036	0.25	1	0.03	0.5221	0.1375
VJR-TRCASH037	1	8	0.25	0.4616	0.1479
VJR-TRCASH038	1	2	0.06	0.0864	0.0349
VJR-TRCASH039	1	1	0.06	0.0354	0.0256
VJR-TRCASH040	4	2	0.06	0.8826	0.1516
VJR-TRCASH041	0.5	2	0.06	1.4946	0.1421
VJR-TRCASH042	>16	2	0.12	0.0235	0.0245
VJR-TRCASH043	1	1	0.03	0.0277	0.0132
VJR-TRCASH044	1	2	0.06	0.1366	0.0321
VJR-TRCASH045	2	2	0.06	0.5184	0.1342
VJR-TRCASH046	0.5	2	0.01	0.1114	0.0359
VJR-TRCASH047	1	2	0.06	0.0630	0.0619
VJR-TRCASH048	1	1	0.06	1.1475	0.1767
VJR-TRCASH049	0.25	2	0.03	1.3264	0.1137
VJR-TRCASH050	0.25	2	0.01	0.5974	0.1137
VJR-TRCASH051	0.25	2	0.03	0.2423	0.1066
VJR-TRCASH052	0.25	1	0.06	1.5814	0.1059
VJR-TRCASH053	0.5	2	0.06	0.8667	0.0858