A STUDY ON *Trichosporon* ISOLATION, ITS MOLECULAR IDENTIFICATION, CLINICAL MANIFESTATIONS AND RISK FACTORS

By

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Dissertation Submitted In Partial Fulfillment Of The Requirements For The Degree of Master of Pathology (Microbiology)

SCHOOL OF MEDICAL SCIENCES

UNIVERSITI SAINS MALAYSIA

2018

ACKNOWLEDGEMENTS

All praises to Allah S.W.T The Most Gracious and The Most Merciful for giving me strength and courage to complete my thesis on time.

I would like to express my deepest gratitude and appreciation to my supervisor Associate Professor Dr Azian Harun for her guidance, unfailing support and for her kind patience and precious time given to me to complete the study. Also thanks to mycology lab staffs Puan Roziawati Yusof and Puan Rosmaniza Abdullah in helping me collecting and keeping the isolates needed throughout the study. My appreciation also goes to post-graduates students such as Yasmin and others that had helped me with lab work especially the molecular part.

My sincere thanks to my colleagues Humairah, Faizah, Norazizah and especially Mahirah, for lending a helping hand and continuous support be it technically or morally from beginning till end of this project. May all of us succeed in our becoming final exam.

My deepest appreciation goes to my husband Mohd Aminuddin Mohd Yusoff who gave me tremendous support throughout this journey. To my son Ahmad Akiff Rafiqi and daughter Amalynn Insyirah who inspired me to finish what I started with their smiles and laughter. My gratitude also goes to beloved mother Wan Ubaidah Omar and also parents in laws, siblings and friends who were always being there to support me.

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ABBREVIATIONS

API	: Analytical Profile Index
BLAST	: Basic Local Alignment Search Tool
CSF	: Cerebrospinal Fluid
CVC	: Central Venous Catheter
DNA	: Deoxyribonucleic acid
ICU	: Intensive Care Unit
IDSA	: Infectious Disease Society of America
ITS	: Internal Transcribed Spacer
IGS	: Intergenic Spacer
HIV	: Human Immunodeficiency Virus
HUSM	: Hospital Universiti Sains Malaysia
MALDITOF	: Matrix-Assisted Laser Desorption/Ionization
MLST	: Multilocus Sequence Typing
OR	: Odds Ratio
PCR	: Polymerase Chain Reaction
PFGE	: Pulsed-field Gel Electrophoresis
RAPD	: Random Amplified Polymorphic DNA
RFLP	: Restriction Fragment Length Polymorphism
rDNA	: ribosomal DNA
rRNA	: ribosomal RNA
rpm	: Revolution per minute
SDA	: Sabouraud Dextrose Agar
Taq	*: Thermus aquaticus*
TBE	: Tris-Borate EDTA
UTI	: Urinary Tract Infection

ABSTRAK

Jangkitan *Trichosporon* adalah jangkitan oportunis yang boleh mengancam nyawa seseorang jika mempunyai faktor risiko seperti kurang ketahanan imuniti. Kaedah konvensional untuk mendiagnosis jangkitan *Trichosporon* adalah tidak mencukupi jika dibandingkan dengan kaedah molekular dari sudut pengenalpastian spesies. Kami mengkaji 155 pesakit yang dijangkiti *Trichosporon* di hospital dari segi bilangan, manifestasi klinikal, kesan klinikal dan faktor risiko untuk mendapat jangkitan *Trichosporon*. Kami mendapati bahawa kebanyakan spesies adalah *T. asahii* (50.3%) dan majoriti berasal dari sampel air kencing (84.5%). Antara faktor-faktor risiko jangkitan *Trichosporon* secara keseluruhan ialah kemasukan ke ICU (95% CI 1.70, 10.37, p = 0.002) dan penerima rawatan antikulat (95% CI 0.06, 0.56, p = 0.003). Bagi kes jangkitan saluran darah, terdapat kaitan yang signifikan dengan penyakit kanser darah (p = 0.001), penerima rawatan antikulat (p = 0.002) dan juga kematian (p = 0.036). Kematian lebih banyak dilihat dalam jangkitan salur darah berbanding dengan keseluruhan jangkitan (55.6% vs 22.5%). Kami juga melakukan kaedah pengenalpastian molekular pada 39 isolat *Trichosporon* yang sedia ada (36 *T. asahii*, dua *T. mucoides* dan satu *T. inkin*) menggunakan jujukan DNA ITS1/4 selepas kaedah konvensional digunapakai. Kami mendapati empat percanggahan keputusan antara kaedah jujukan DNA dengan kaedah konvensional di mana dua *T. asahii* dikenalpasti sebagai *T. montevideense* dan dua *T. mucoides* adalah *T. debeurmannianum* oleh jujukan DNA. Kesimpulannya, jangkitan *Trichosporon* pada pesakit boleh menyebabkan risiko kematian yang tinggi dan keperluan kaedah pengenalpastian molekular adalah penting terutamanya dari segi pengenalpastian spesies utuk pemberian rawatan yang tepat.

ABSTRACT

Trichosporon infection is a rare opportunistic, life threatening mycosis in patients with predisposing factors such as underlying immunodeficiency. Conventional methods to diagnose trichosporonosis are inadequate compared to molecular methods in terms of species identification. We reviewed 155 patients with *Trichosporon* isolations in hospital to study the proportion, clinical manifestations, outcome and risk factors for having *Trichosporon* infections. We found out that the majority species isolated were *T. asahii* (50.3%) and mostly were from urine samples (84.5%). Among risk factors for overall *Trichosporon* infections were ICU admission (95%CI 1.70, 10.37, p=0.002) and antifungal recipients (95%CI 0.06, 0.56, p=0.003). For *Trichosporon* fungemia, there were significant associations among those with hematological malignancy (p=0.001), antifungal recipients (p=0.002) and mortality (p=0.036). Mortality was observed more in fungemia compared to overall infection (55.6% vs. 22.5%). We also performed molecular identification on 39 available *Trichosporon* isolates (36 *T. asahii*, two *T. mucoides* and one *T. inkin*) by ITS1/4 sequencing that were previously identified by conventional method. We observed four discrepancies by conventional method where two *T. asahii* were identified as *T. montevideense* and two *T. mucoides* were *T. debeurmannianum* by ITS sequencing. In conclusion, *Trichosporon* infection in susceptible patients posed high mortality risk which required molecular identification for species identification to guide proper therapy.

1.1 Background

Trichosporon spp. are the causative agents of a spectrum of fungal diseases ranging from mild cutaneous mycoses in immunocompetent hosts to fatal disseminated diseases in severely immunocompromised patients. They are basidiomycetous yeast-like anamorphic organisms that are widely distributed in nature and found predominantly in tropical and temperate areas. *Trichosporon* spp. can be found in environment including soil, water, wood, and vegetables, rivers, lakes, seawater, cheese, scarab beetles, bird droppings, bats, pigeons, and cattle.[1,2] *Trichosporon* colonized 11% of 1,004 healthy male volunteers on their normal perigenital skin eg. scrotal, perianal, and inguinal sites of the body.[3] Although most *Trichosporon* spp. routinely isolated in laboratories are related to episodes of colonization or superficial infections, this fungus has been recognized as an emerging opportunistic agent causing invasive infections in tertiary care hospitals worldwide.[4]

1.1.1 History

In 1865, Beigel designated genus *Trichosporon* when he observed this microorganism causing a benign hair infection. The word *Trichosporon* was derived from Greek words which carry the meaning of *Trichos* (hair) and *sporon* (spores). This nomenclature was related to the presence of irregular nodules along the body and head hair, consisting of a fungal infection, which was further characterized as 'white piedra' (piedra means "stone" in Spanish). In 1902, all *Trichosporon* species were designated as *Trichosporon beigelii,* an arthrospore-containing yeast. *Trichosporon cutaneum* was later being found from fungal elements collected from a patient with pruritic lesion in 1926. Nevertheless, scientists had considered *T. beigelii* and *T. cutaneum* to be the same species. This directed to the usage of two names for this fungus with clinical relevance; *Trichosporon beigelii,*

preferred by physicians, and *Trichosporon cutaneum*, favored by environmental mycologists.[5]

1.1.2 Taxonomy

Over the past decade, the taxonomy of the genus *Trichosporon* has been subjected to extensive amendment. New taxonomic concept was proposed based on molecular analysis and the name *T. beigelli* has been replaced.[5] Currently the genus contains approximately 50 species including few medically important *Trichosporon* species: *T. asahii, T. asteroides, T. cutaneum, T. inkin, T. mucoides and T. ovoides and T. montenvideense.*[1,6,7] *T. asahii, T. mucoides* and *T. cutaneum* are the main agents for invasive infection while *T. cutaneum, T. inkin* and others have been associated with superficial infections.[1]

1.2 Clinical significance

Trichosporon spp. are medically important basidiomycetous yeasts which can be found in environment and become part of the human mycobiota, able to colonize and proliferate in different parts of human body, including the gastrointestinal system, respiratory tract, skin, and vagina. This yeast-like pathogen may cause deep-seated, mucosa-associated, or superficial infections. Invasive *Trichosporon*osis has been acknowledged predominantly in patients with immunosuppression, while superficial infections and allergic pneumonia are documented mainly in healthy hosts.[1]

Trichosporon asahii is the most important etiologic agent of invasive disease and is responsible for 88% of invasive trichosporonosis in immunocompromised patients. [8,9] It was frequently isolated in urine specimens of ICU patients.[10] Many of *Trichosporon* invasive infections have been described in hematological malignancies patients, which occurred during neutropenic phases[9,11], with more than half of bloodstream infections being catheter-related. [10,12] *Trichosporon* has been recognized as a colonizer and must be kept under control, particularly in neutropenic patients.[13] *Trichosporon* infection and colonization seems to be related to catheter-use, which begins with initial invasion of mucosal barrier followed by vascular invasion and dissemination to other sites.[14] The presence of invasive devices is a significant factor, owing to the ability of *Trichosporon* spp. to form biofilm. Major contributing factor of persistent infection is the ability to form biofilms particularly in *T. asahii* [15], which may result in more severe clinical course since the organism is protected from the host's immune system.

1.3 Laboratory identification of *Trichosporon*

1.3.1 Conventional identification methods

The genus *Trichosporon* is phenotypically characterized by the ability to form blastoconidia, true mycelia, and, most importantly arthroconidia, asexual propagules that disarticulate from true hyphae. Mycelium is the vegetative part of a fungus or fungus-like bacterial colony, consisting of a mass of branching, thread-like hyphae. Blastoconidia is the unit of asexual reproduction produced by budding. Arthroconidia is a type of fungal spore typically produced by segmentation of pre-existing fungal hyphae (Figure 1.1). *Trichosporon* spp. are able to utilize different carbohydrates and carbon sources and able to degrade urea. Most *Trichosporon* isolates grow within 36-72 hours on blood agar and most non-selective primary isolation culture media. Cell cultures grow on Sabouraud

dextrose agar (SDA) as yeast colonies with colours ranging from white to cream, typically displaying aspects such as cerebriform and radial surfaces (Figure 1.1). Colonies may become dry and membranous with age. Culture identification of *Trichosporon* spp. from clinical specimens is often confused with *Candida* spp., which can be differentiated by the ability of *Trichosporon* spp. to produce arthroconidia and hydrolyze urea.

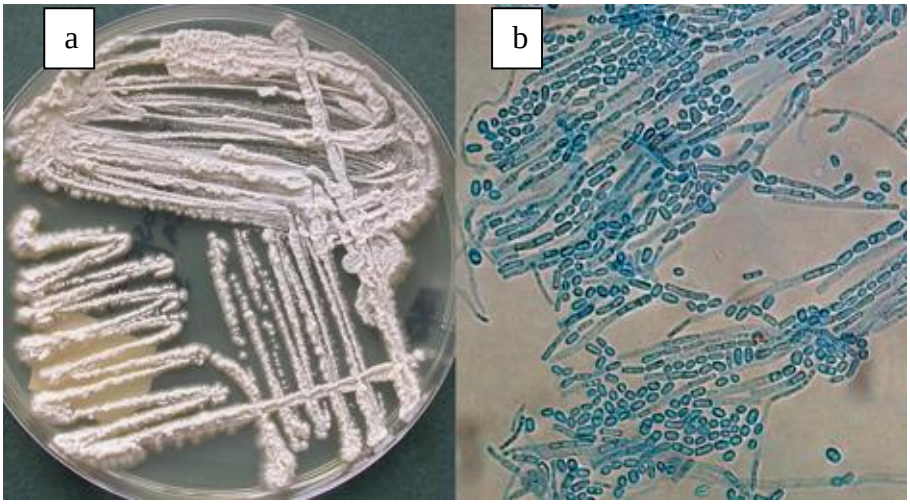


Figure 1.1: Pictures of *Trichosporon asahii* (a) Colonies of *T. asahii* with raised and waxy appearance, which develop radial furrows and irregular folds; (b) Arthroconidia of *Trichosporon*.

(Adapted from http://www.mycology.adelaide.edu.au/images/trichosporon-asahii.jpg)

1.3.2 Commercial kits for fungal identification

A few commercial non-automated and automated systems have been used for the identification of *Trichosporon* spp. even though many of these methods do not include new taxonomic categories in their databases.[1] Therefore, the identification of the genus *Trichosporon* is simplified due to incomplete databases and classification keys.

Basically the commercial methods were based on the evaluation of the ability of the fungus to assimilate carbon sources, organic acids and observation of enzymatic profile. The most commonly used non-automated commercial methods for yeast identification in clinical laboratories include API 20C AUX (bioMérieux, Mercy l'Etoile, France), ID 32C (bioMérieux, Mercy l'Etoile, France) and RapID Yeast Plus system (Innovative Diagnostic System, Norcross, GA, USA). The most commonly used automated systems for yeast identification in clinical laboratories are Vitek Systems (bioMérieux, Vitek, Hazelwood, MO, USA), and Baxter Microscan (Baxter Microscan, West Sacramento, CA, USA). In HUSM, API 20C AUX is currently used and the present database only include three species; *T. asahii, T. inkin* and *T. mucoides.* There have been a number of published reports addressing the accuracy of commercial yeast identification methods which provide limited data on specific *Trichosporon* species.[1]

1.3.3 Molecular methods

In the recent years, molecular techniques such as DNA sequencing have been shown to provide more accurate and consistent fungal identification up to species level. Given their high specificity, the methods are highly discriminative even for closely related species.[16]

1.3.3.1 Fungal DNA sequencing

Fungal DNA sequencing offers accurate and reliable identification of fungal species based on specificity of selected genes sequences which enable interspecies differentiation. Various genes have been used for fungal identification such as ribosomal RNA (rRNA) and elongation factor 1-alpha.[17,18] Ribosomal RNA genes are the most frequently and widely utilised target for fungal identification by DNA sequencing. Fungal

rRNA genes are tandemly repeated, with each repeat encoding 18S (small-subunit), 5.8S, and 26S (large-subunit) genes. Two other regions exist in each repeat: the internal transcribed spacer (ITS) region and the intergenic spacer (IGS) region (Figure 1.2). The D1/D2 regions of 26S and ITS sequences have been used mainly to identify pathogenic fungi. At present, the 26S rDNA sequences of almost all yeasts, including non-pathogenic species, have been determined[19] and the analysis of ITS sequences has been carried out mainly for pathogenic yeast species.[20]

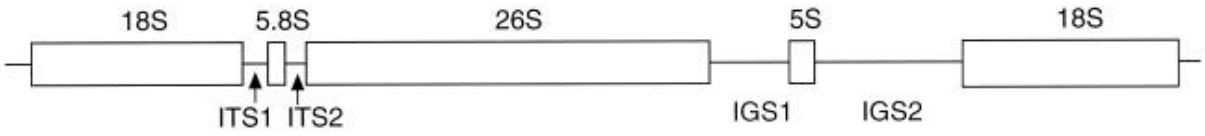


Figure 1.2: Schematic representation of the rDNA locus in *Trichosporon*. Boxes indicate coding regions. (Reproduced from Sugita *et al*, 2002).

1.3.3.2 ITS Sequencing

Molecular identification of *Trichosporon* can be achieved by sequencing of ITS region of rDNA. [21,22] Sugita concluded that the six medically relevant species could be accurately identified by their ITS sequences. Nevertheless, analysis of sequences of the intergenic spacer region (IGS1), which is located between the 26S and 5S genes of rDNA, suggested higher variations in the IGS1 region than that of ITS region, thus allowing more accurate *Trichosporon* identification. [23] Fungal genotyping can be performed to assess the genetic relatedness among the strain, which is especially important for epidemiological study as well as infection control purposes. Genotyping techniques include many methods such as random amplification of polymorphic DNA (RAPD), pulse field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP) analysis and DNA sequence-

based method such as multilocus sequence typing (MLST).[24] Methods based on the polymerase chain reaction (PCR) undoubtedly show a higher sensitivity than culture, with a comparable efficiency.

1.4 Treatment

Definitive antifungal treatment for invasive trichosporonosis is not yet well established. Few studies documented excellent *in vitro* activity of newer generation azoles including voriconazole and posaconazole, but high *in vitro* resistance to amphotericin B and fluconazole[1]. Echinocandins despite being the empiric treatment for neutropenic fever [25], seem to be the least effective agents especially in patients with hematologic malignancies.[8,9] Thus, clinicians need to be aware of this organism, as trichosporonosis clinically may appear similar to disseminated candidiasis.[26]

1.5 Rationale of the study

Regardless of the growing attention on fungal infections and their impacts, little is known about the current epidemiology of *Trichosporon* such as proportion, clinical manifestations and risk factors particularly in a local Malaysian setting. Despite the knowledge that it has been considered as one of the most important evolving causes of invasive fungal infection in immunocompromised patients, data on its course and evolution is still scarce.

This study would benefit and evoke clinicians to become alert to the prevalence of *Trichosporon* infection especially in immunocompromised patients with high risk factors,

such as haematological malignancy patients, patients who are on prolonged antibiotic use, immunosuppressive treatment, diabetes mellitus and prolonged ICU stay. In a few data gathered regarding *Trichosporon* isolation in Malaysia, only two cases were reported involving non-malignancy patients with a case of recurrent glossitis[27] and subcutaneous infection[28], despite of it being frequently reported worldwide. As for the treatment, trichosporonosis may be suspected if the patients with yeast infections do not respond to standard antifungal treatment particularly in patients with hematological malignancies and neutropenic fever. It is known that in empirical treatment for yeast infections prior to species identification, echinocandins are the primary choice as clinicians tend to think of *Candida* infection.[29,30] Unfortunately, echinocandins are not recommended and should not be used for treatment of trichosporonosis.[2,9] Early diagnosis of *Trichosporon* infection is important as appropriate antifungal treatment could have been initiated much earlier and poor clinical outcome can be prevented.

Conventional methods of identification of *Trichosporon* species are often inconclusive and time-consuming. Besides, biochemical identification techniques with poor database are among the common techniques used for diagnosis for past 10 years.[31] It is understood why most of the reports only referred to the genus *Trichosporon* with no species determination. In some cases, clinical isolates were merely identified as *T. asahii* or *T.* non-*asahii*. The lack of accurate laboratory tools for complete identification of *Trichosporon* strains in routine laboratories impairs the understanding of epidemiological and clinical peculiarities as well as differences in terms of clinical response to conventional antifungal therapy. Thus, early and accurate diagnosis remains a challenge whenever appropriate and timely antifungal treatment is needed as poor clinical outcome can be prevented. In this present study, molecular identification technique has been

applied for accurate species identification of *Trichosporon* and the expected discrepancy with the previous conventional identification has been demonstrated.

1.6 Study objective

1.6.1 General Objectives

To study the proportion and molecular identification of *Trichosporon* spp., the clinical manifestations of their infections and the risk factors for their isolation and infections in hospitalized patients.

1.6.2 Specific Objectives

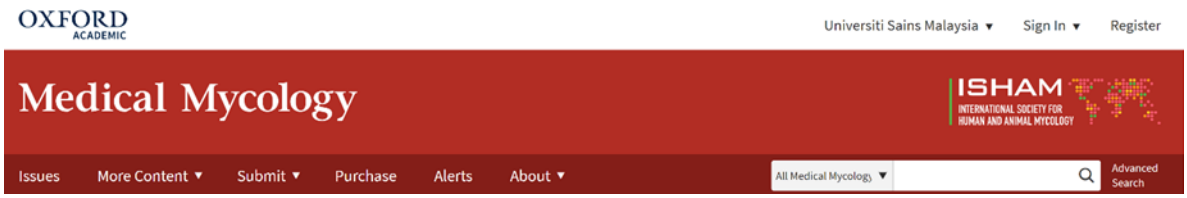
1. To determine the identity of *Trichosporon* spp. Isolated from clinical samples by molecular method.
2. To determine the proportion of *Trichosporon* spp. isolated from clinical samples
3. To describe clinical manifestation and outcome of *Trichosporon* infections
4. To determine the risk factors associated with *Trichosporon* colonization and infection

2.1 Manuscript

Title: Molecular identification, clinical manifestations and risk factors of *Trichosporon* species isolated from a tertiary centre in northeastern Malaysia.

Authors: Wan Amani Wan Abdul Azim, Roziawati Yusof, Azian Harun.

(Manuscript as per submission to the journal Medical Mycology)



Original Article

Molecular identification, clinical manifestations and risk factors of *Trichosporon* species isolated from a tertiary centre in northeastern Malaysia.

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Keywords: *Trichosporon*, ITS, identification, fungemia

Abstract

Trichosporon infection is a rare opportunistic, life threatening mycosis in patients with predisposing factors such as underlying immunodeficiency. Conventional methods to diagnose trichosporonosis are inadequate compared to molecular methods in terms of species identification. We reviewed 155 patients with *Trichosporon* isolations in hospital to study the proportion, clinical manifestations, outcome and risk factors for having *Trichosporon* infections. We found out that the majority species isolated were *T. asahii* (50.3%) and mostly were from urine samples (84.5%). Among risk factors for overall *Trichosporon* infections were ICU admission (95%CI 1.70, 10.37, p=0.002) and antifungal recipients (95%CI 0.06, 0.56, p=0.003). For *Trichosporon* fungemia, there were significant associations among those with hematological malignancy (p=0.001), antifungal recipients (p=0.002) and mortality (p=0.036). Mortality was observed more in fungemia compared to overall infection (55.6% vs. 22.5%). We also performed molecular identification on 39 available *Trichosporon* isolates (36 *T. asahii*, two *T. mucoides* and one *T. inkin*) by ITS1/4 sequencing that were previously identified by conventional method. We observed four discrepancies by conventional method where two *T. asahii* were identified as *T. montevideense* and two *T. mucoides* were *T. debeurmannianum* by ITS sequencing. In conclusion, *Trichosporon* infection in susceptible patients posed high mortality risk which required molecular identification for species identification to guide proper therapy.

Introduction

Fungal infections have been significantly increasing for the past few decades because of the expanded population of immunosuppressed patients resulting from advances in medical technology such as aggressive chemotherapy, transplantation, HIV infection, and malignancies.[1,2] *Trichosporon* is among the emerging causative agents of mycoses causing life threatening fungal infections in these patients. It is the second most common fungal pathogen isolated after *Candida*[3,4] in hematological malignancies with mortality rates ranging between 60-80% despite administration of antifungal therapy.[5] In the last years, cases of *Trichosporon* infections were increasingly reported mainly in hematological malignancy patients due to the growing apprehension on this yeast and upgraded clinical diagnostic methods for medical mycology.[6]

Over the past decade, the taxonomy of the genus *Trichosporon* has been subjected to extensive amendment. Previously all pathogenic members of genus *Trichosporon* was known as *Trichosporon beigelli*[7], an environmental and saprophytic fungus responsible for fungal infection of hair shaft named 'white piedra'.[8] New taxonomic concept was proposed based on molecular analysis and the named *T. beigelli* has been replaced.[7,9] Currently the genus contains approximately 50 species including a number of medically important *Trichosporon* species: *T. asahii, T. asteroides, T. cutaneum, T. inkin, T. mucoides, T. ovoides* and *T. montevideense.* [1,10,11] *T. asahii, T. mucoides* and *T. cutaneum* are the main agents for invasive infection while *T. cutaneum*, *T. inkin* and others are associated with superficial infections.[1]

Culture identification of *Trichosporon* spp. from clinical specimens are often confused with *Candida* spp. with the main differences being arthroconidia production and ability to hydrolyze urea in *Trichosporon* spp.. Conventional identification methods for species of *Trichosporon* are often inconclusive and time-consuming. Moreover biochemical identification systems, which are the common techniques used for diagnosis for past 10 years[6], have limited databases to allow accurate identification. Thus, early and accurate diagnosis remains a challenge whenever appropriate and timely antifungal is needed in order to prevent poor clinical outcome in significant infections. Molecular technique by nucleotide sequences is more accurate and consistent to give identification to species level with high sensitivity and specificity and being discriminative even for closely related species.[12] Molecular assays using interspacer region (ITS) genes of rDNA as target have been the most frequently described.[13,14]

A study was conducted at the Hospital Universiti Sains Malaysia, a tertiary hospital in Kelantan state located at the east coast of Peninsular Malaysia, to determine the proportion, demographics and clinical characteristics of patients with *Trichosporon* isolated from clinical specimens. The risk factors for being infected by *Trichosporon* spp. were identified. We also determined the species identification of *Trichosporon* spp. using molecular method.

Methods

Trichosporon isolates and data collection

Trichosporon species isolated from clinical samples in hospitalized patients at Hospital Universiti Sains Malaysia from 2008 to 2016 were collected. The isolates were identified based on standard phenotypic methods, including typical morphological appearance of white yeast-like colonies and production of arthroconidia and pseudohyphae. Further identification was performed by assimilation of organic substrates using API 20C AUX (bioMerieux, France) in accordance to manufacturer's instruction. All isolates were stored at the Mycology Laboratory, Department of Medical Microbiology and Parasitology, School of Medical Sciences, Universiti Sains Malaysia. Medical records were retrieved and reviewed, and patients' data including demographic, clinical manifestation, risk factors and outcomes were recorded. Patients were divided into colonization or infection groups according to clinical findings. This study has been approved by The Human Research Ethics Committee of Universiti Sains Malaysia (Reference no. USM/JEPeM/15120527).

Definitions

Since clinical features of *Trichosporon* infections could not be distinguished from the ones due to *Candida*[3], some of case definitions used in this study were adapted from Kourkoumpetis *et al.*[15] Colonization was defined as the presence of a positive culture from a non-sterile site without clinical signs of infection or with signs of infection clearly attributed to other microorganism. Infection was determined by isolation of *Trichosporon* with fungemia, pulmonary infection, urinary tract infection (UTI) and other infections when other causes have been excluded. *Trichosporon* fungemia was defined as isolation of *Trichosporon* from the bloodstream in at least one blood culture in patients having

signs and symptoms of infection. Pulmonary infection was defined as recovery of *Trichosporon* species from sputum in absence of other pathogens causing opportunistic infections.[16] Urinary tract infection was defined as the presence of significant amount $\geq 10^5$ colony-forming unit/ml of *Trichosporon* yeast cells in patients without any co-isolation of other bacterial pathogens, plus significant pyuria and clinical symptoms cannot be attributed by other etiology.[17]

DNA extraction

DNA extraction was performed as previously described with some modification.[18] In summary, 500µL of lysis buffer and 5µL of 2-mercaptoethanol (Sigma-Aldrich, USA) were added to frozen *Trichosporon* colonies. The mixture was vortexed vigorously and incubated at 65ºC for one hour. Then, 500µL of phenol-chloroform-isoamyl alcohol (25:24:1) (Sigma-Aldrich, USA) was added, mixed thoroughly and centrifuged at 10000rpm for 10 minutes. The top aqueous layer was removed and placed into a new tube. An equal amount of cold isopropanol was added and genomic DNA was allowed to precipitate for at least two hours at -20ºC. The tube was then centrifuged at 10000rpm for 10 minutes at 4ºC. The resulting DNA pellet was washed twice with 500µL of 70% ethanol. The pellet was dried and finally reconstituted in 100µL of sterile deionized water.

PCR analysis and sequencing

The ITS1, 5.8S, and ITS2 regions of the rDNA gene cluster were amplified with the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG) and ITS4 (5'-TCCTCCGCTTATTGATATG) as described previously.[19] The PCR product was commercially sequenced in both directions by using the same amplification forward and reverse primers.

Statistical analysis

Statistical analysis were performed using SPSS 24 statistical software. Demographic and clinical characteristics of patients with *Trichosporon* spp. isolation were analyzed using Pearson's chi squared test when met assumption or Fisher's exact test when expected count of less than five is more than 20% (only for categorical variables). Logistic regression was performed by simple logistic regression, followed by multivariate analysis for multiple logistic regression to determine the factors associated with the occurrence of *Trichosporon* infection. Based on simple logistic regression, variables with p-value of <0.25 were included to multiple logistic regression. Association factors based on specific infection due to *Trichosporon* fungemia were also performed using Fisher's exact test.

Results

Proportion and patient characteristics

Records of 155 patients with positive *Trichosporon* cultures from year 2008 to 2016 were reviewed. Sources of *Trichosporon* isolation were shown in Figure 2.1. Majority of *Trichosporon* were isolated from urine specimens (n=131, 84.5%) and only 26 (19.8%) were considered as true infection, while the rest (n=105) were colonizers. Nine positive cultures (5.8%) were isolated from blood which were all shown true infection. Other samples included; eight from pus with only one considered as true infection, two from sputum (both were true infection) and two from body fluid which were colonizers, and one each from high vaginal swab, tissue and nail which were all included in infection group. According to type of species distribution (Figure 2.2), *T. asahii* was found in 78 of 155 (50.3%) patients, but without significant difference between both infection and colonization groups. *T. mucoides* was isolated in six patients (3.9%) while *T. inkin* only in three patients (1.9%). In 68 (43.9%) patients, the isolates were previously identified only to genus level and reported as *Trichosporon* spp..

The patients included in this study were 73.5 % male (114 out of 155) and the mean age was 52 years old. *Trichosporon* was isolated from most patients admitted for acute coronary syndrome or cardiac complication (21.9%), pneumonia (16.1%) and sepsis (14.8%) which were among of the commonest diagnosis for hospital admission. About 87.7% of patients received antibiotics at the point of *Trichosporon* isolation, while 12.9% was given antifungal therapy either for prophylaxis, empirical or targeted therapy.

Table 2.1 showed comparisons between the infection and colonization groups. Among the 155 patients, 40 (25.8%) fulfilled criteria for infection and the remaining 115 (74.2%) were categorized as colonization or cultures of uncertain significance. There were no significant difference in age between both groups (p=0.487).

Risk factors

The risk factors for *Trichosporon* infection were shown in Table 2.2. In univariate analysis, the factors associated with *Trichosporon* infection were ICU admission (95%CI 1.41, 6.76, p=0.005), antibiotics recipients (95%CI 0.02, 1.07, p=0.058) and antifungal recipients (95%CI 0.11, 0.75, p=0.011). Based on simple logistic regression, variables with p- value of <0.25 were subjected to multiple logistic regression. In multivariate analysis, independent risk factors associated with *Trichosporon* infection were ICU admission (95%CI 1.70, 10.37, p=0.002) and antifungal recipients (95%CI 0.06, 0.56, p=0.003). For *Trichosporon* fungemia, there were statistically significant associations when having hematological malignancy (p=0.001), febrile neutropenia (p<0.001), antifungal recipients (p=0.002) and death as outcome (p=0.036) using Fisher's exact test (Table 2.3).

Molecular identification

Molecular identification was performed on any viable *Trichosporon* spp. isolated from year 2008-2016. A total of 39 isolates were revived and re-identified by API 20C AUX out of which 36 isolates were identified as *T. asahii*, two as *T. mucoides* and one as *T. inkin*. All 39 isolates were subjected to molecular identification. Out of 36 isolates identified as *T. asahii* by API 20C AUX, two were discrepant and identified as *T.*

montevideense by ITS sequencing. Both *T. mucoides* were identified as *T. debeurmannianum* by molecular method. Four isolates that gave discrepant results signified new species never before seen in our lab.

Discussion

Trichosporon spp. are medically important basidiomycetous yeast-like organism which can be found in natural environment including soil, water, wood, vegetables[1,5] and become part of the human mycobiota. *Trichosporon* is able to colonize diverse parts of human body, including the gastrointestinal system, respiratory tract, skin, and perigenital region[20]. This yeast-like pathogen may cause deep-seated, mucosa-associated or superficial infections. Invasive trichosporonosis has been documented mostly in patients with immunosuppression, whereas superficial infections and allergic pneumonia are found predominantly in immunocompetent hosts.[1]

Most of *Trichosporon* isolated from hospital were *Trichosporon asahii* [1,6,10], which is in line with our current study. The proportion *T. asahii* could have been higher since not all isolates were available and identified to species level during the study period. In this study, urine made the biggest source of *Trichsoporon* isolation, whereby *T. asahii* was the most isolated species (51.9%), which also concurred with previous studies. [20,21] It has been reported that *T. asahii* was the commonest cause of colonization in urine and catheters of hospitalized patient, whereas *T. cutaneum* was the major species isolated from normal perigenital skin.[20] Risk factors for colonization included indwelling/endotracheal catheter, diabetes mellitus and old age.[21] Significant risk is seen when patients were admitted to ICU, with 4.2 times the odd to have *Trichosporon*

infection compared to no ICU admission (95% CI 0.06, 0.56, p=0.003). The prevalence of *Trichosporon* spp. in urine cultures in ICU patients is approximately 6%.[22] Indwelling urinary catheter was found in 69% of our patients, which is comparable to another study where urethral catheter was found in 60.8-77.6% subjects with funguria.[23,24] However there was no significant difference in the presence of urinary catheter between the patients with true infections and those in colonization group.

Trichosporon is also among significant causes of rare fungemia, which accounted for 25% of non-*Candida* yeasts isolated from blood of patients with cancer.[25] *T. asahii* is one of the most important etiologic agents for trichosporonemia and responsible for 88% of invasive trichosporonosis in immunocompromised patients.[16,26] Most non-*Candida* or rare invasive yeast infections including *Trichosporon* infections involved patients in hematology and ICU settings.[27] This study is also in agreement with other previous studies with regard to the risks of having trichosporonemia in hematological malignancies patients particularly during neutropenic phase.[8,16] Nevertheless, we did not find catheter-related as a risk for trichosporonemia, as previously reported in other studies.[6,25,28,29] A recent largest review on *Trichosporon* fungemia found that patients with neutropenia failed to recover from infection despite antifungal therapy unless with removal of the catheters.[6] This is also consistent with the Infectious Diseases Society of America (IDSA) recommendation of central venous catheter removal in cases of candidemia.[30] Major contributing factor of persistent infection especially in *T. asahii* is the ability to form biofilms[31] which can have more severe clinical course since they are protected from the host's immune system. The mortality rate among subjects with positive cultures from blood was 55.6% which is significant (p=0.036). Other previous studies[25,28] also reported similar findings with some reported as high as 80%.[2,5] The mortality rate of

trichosporonemia had been reported to be consistently higher than candidemia[3,25], probably due to earlier appropriate therapy intervention by clinicians in candidemia.

In most settings worldwide, culture has been the only diagnostic method for early diagnosis of *Trichosporon.* Phenotypically, *Trichosporon* spp. can produce blastoconidia, arthroconidia, pseudohyphae, and true septate hyphae. When arthroconidia is visualized, it is recommended to do urease test to differentiate *Trichosporon* from phenotypically identical organism *Geotrichum.*[1] On Sabouraud dextrose agar, the colonies appear as white to cream color, with distinctive cerebriform, wrinkled, verrucosed and radial surfaces, and subsequently become dry and membranous with age.[32,33] Several commercial non-automated systems (for example; API 20C AUX) and automated systems (Vitek 2 ID YST) have been used for identification. In this study, all isolates were identified as *Trichoporon* spp. by API 20C AUX. Using molecular method, we then observed identification discrepancies in four isolates, which included species that have never been isolated in our lab, *T. montevideense* and *T. debeurmannianum*. Although rare, *T. montevideense* is known as pathogenic *Trichosporon* [11,34,35] which have been isolated from blood and urine sample. *T. debeurmannianum,* which was recently re-grouped to *Trichosporon* species from *Cryptococcus humicola* complex[36], was isolated from urine and might be associated with contamination. The discordant results were partly because the current database for identification systems like API 20C AUX contain only three main species; *T. asahii*, *T. inkin* and *T. mucoides.*[1,26,37] The system databases did not take into account the new taxonomic categories, thus leading to possible misidentification and discrepancies. The accuracy of commercial yeast identification methods was being addressed in most papers, which lead to the demand for reliable diagnostic modalities including the usage of molecular methods for more accurate and rapid

identification.[12,37,39] Specific diagnostic antigen detection by serological methods for example β-D-glucan has limited use for trichosporonosis.[1,27]

Species identification based on nucleotide sequencing is more accurate and consistent to give identification at species level compared to conventional method. Sugita *et al*. had sequenced and analyzed the interspacer region (ITS1 and ITS2) genes of rDNA from *Trichosporon* species, subsequently after his previous work on small subunit region sequences amplified by primer pair TRR and TRF.[13,14] The main advantages of molecular methods are their high sensitivity and specificity and their being fully discriminative even for closely related species.[12] Despite being promising as rapid, easy, and cost-effective, few strains with ITS region sequences showed high similarity to any groups of related species, with more discriminatory power shown by higher variation of IGS1 region.[40,41] However not all clinical laboratories use IGS1 sequencing options for routine analysis and it is used for genotyping rather than species identification.[41] Fungal genotyping is also useful to explore population dynamics and transmissibility in clinical setting, thus enabling identification of possible outbreak.[41,42] A study in neighboring country Thailand revealed that types I and III were predominant out of five *T. asahii* genotypes from IGS region. This finding differs from findings from other countries, suggesting that there is a geographic substructure among *T. asahii* clinical isolates.[43] Genotype III of *T. asahii* has been shown to be associated with higher pathogenicity due to higher hemolytic and biofilm formation activities.[43] Nevertheless the rising application of other modalities like Matrix Assisted Laser Desorption/Ionization Time of Flight (MALDI TOF) mass spectrometry as a cheaper alternative to gene sequencing is overwhelming and has been proven to be an excellent diagnostic tool that provided reliable identification with good discriminatory power for *Trichosporon* species and other clinically important fungi. [44,45]

From our observation, antibiotics usage were prominent in infection group especially when organism was not suspected to be yeast. Antibiotic recipients have high association factor in this study (p=0.027), which is consistent with a previous study on *Trichosporon* funguria.[22,24] Empirical or prophylactic antifungal therapy started in persistent infections was perceived as protective risk by our study (95%CI 0.06, 0.56, p=0.003). Echinocandin is the initial choice for empirical treatment as clinicians tend to think of *Candida* infection.[2,30,46-48] Despite being the empirical treatment for neutropenic fever[30,49], echinocandin seems to be the least effective against *Trichosporon* infection especially in patients with hematological malignancies.[16,26] It is not recommended for treatment of trichosporonosis for the organism's intrinsic resistance to echinocandin.[5,16,39] Published cases proved that major breakthrough infection of *Trichosporon* spp. were related to the use of echinocandin and amphotericin B, which may translate into poor outcome as a result of delayed diagnosis.[6] Evidence for definitive antifungal treatment for invasive trichosporonosis is lacking and has not been established. However, few studies documented excellent *in vitro* activity of newer generation azoles including voriconazole and posaconazole but high *in vitro* resistance to amphotericin B and fluconazole.[1,6,43] Nonetheless voriconazole is not effective once *Trichosporon* exists in biofilm form.[24] The precise identification of *Trichosporon* spp. is vital as *T. asahii* seemed to be more resistant to amphotericin B than triazole, while non- *asahii* tend to be more resistant *in vitro* to triazole agents compared to amphotericin B.[12,41]

In conclusion, early identification of *Trichosporon* remains a challenge particularly at species level and frequently a little too late to permit effective therapeutic intervention. Wide application of a more accurate and rapid molecular method is needed as it is far more superior compared to conventional identification method. Health care providers