International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 13 (2), pp. 001-005, February, 2019. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Trichosporon identification methods for isolates obtained from different clinical specimens

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Accepted 15 January, 2019

***Trichosporon* is a medically important genus that includes the causative agents of both deep- seated, mucosa-associated infection and superficial infection. In this study, we aimed to present data on the phenotypic and molecular identification of Trichosporon species recovered from various clinical specimens representing both superficial and systemic infections. 397 samples (65 blood cultures, 192 nail scrapings, 140 skin scrapings) were included for isolation, phenotypic and genotypic identification of *Trichosporon* species. Cases of hematological malignancies, onychomycosis, and *Tinea pedis* were positive for yeast isolates with percentage of 10.7, 16.6, 20.7%, respectively. Based on the morphologic characters of isolated colonies on Sabouraud dextrose agar (SDA), microscopic examination of colonies on rice agar, and stained smears, yeasts identified as *Trichosporon* were: 9(4.68%) from nail samples, and 5(3.6%) from skin samples and 0(0.0%) from blood cultures. Polymerase chain reaction (PCR) using *Trichosporon* genus specific primers for 68 yeast isolates was positive in 16 samples (14 were previously identified by morphology and 2 nail scrapings were falsely diagnosed negative) at 170 bp. We subsequently performed 2 PCR runs on the identified 16 samples using specific primers for each of *T. asahii* and *T. mucoides*. Their sequences included ITS1, ITS2. They yielded specific amplification of a DNA fragment at 430 bp in 13(16) samples, which is specific for *Trichosporon asahii*, and 0(0.0%) sample was positive for *Trichosporon mucoides* specific primers. Occurance of trichosporonosis is not rare in human. *T. asahii* species is common in our locality. Molecular methods for identification of *Trichosporon* are more precise. The standardization of laboratory methods for *trichosporon* identification and antifungal susceptibility tests are necessary to investigate for both superficial and systemic trichosporonosis.**

Key words: *Trichosporon asahii,* polymerase chain reaction (PCR)*, Trichosporon mucoides.*

INTRODUCTION

Trichosporon is a medically important genus that includes the causative agents of both deep-seated, mucosa-associated infection and superficial infection (Sugita et al., 1999).

Deep- seated trichosporonsis have a high mortality rate, and the prognosis for patients is very poor; *trichosporon* species are also responsible for summer-type hypersensitivity pneumonitis (Sugita et al., 1999).

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Several methods used for *Trichosporon* species identification have been reported, including morphological and biochemical tests and the use of molecular tools. Despite the fact that phenotypic methods are more suitable for routine in general microbiology laboratories, the accuracy for identification seems to be limited (Sugita et al., 1994; Walsh et al., 2004).

The genus *Trichosporon* is characterized by the ability to form arthroconidia, blasatoconidia, hyphae, and pseudohyphae. All species are able to assimilate different carbohydrates and carbon sources and to degrade urea. However significant difference in the results generated by the same organisms tested by different authors can be

observed in regard to biochemical and physiological tests performed with different substrates used in yeast identification keys (Sugita et al., 1995; Pincus et al., 2007).

Up to our best knowledge, there has been no previous study on morphologic and genotypic characterization of *Trichosporon* species in Egypt. In this communication, our aim was to present data on the morphologic and molecular identification of *Trichosporon* species recovered from various clinical specimens representing systemic and superficial infection.

MATERIALS AND METHODS

A total of 397 samples were collected over 2 years (May 2007 to May 2009). Patients of this study were of two groups, first group included 65 patients with different types of hematological malignancies selected from the Oncology Center, Mansoura University Hospitals. Their ages ranged from 21 to 45 years. They consulted for persistent fever in spite of antibiotic therapy for 7 to 10 days before. An oral consent was obtained from each patient. 3 to 5 ml of blood were obtained to perform blood culture. The second patients group included 192 patients with onychomycosis and 140 patients with *Tinea pedis* from the Out Patient clinic of Dermatology, Mansoura University hospital. Nail and skin scrapings were obtained, using sterile scalpels, after cleaning the area with alcohol 70%.

For isolation of yeasts, blood culture was performed by lysis centrifugation technique. 3 to 5 ml heparinaized blood collected under aseptic precautions were subjected to lysis by using sterile D.W then centrifugation. The blood concentrates were cultured on Sabouraud's dextrose agar (SDA), incubated at 37°C for 7 days before discarded as negative. It was mentioned by Reisner and woods (199), that the average recovery time for yeasts and molds using lysis centrifugation methods is 3.8 and 10.5 days, respectively. Nail and skin collected samples were inoculated onto SDA slants with chloramphenicol (50 mg/l) and incubated at 37°C for up to 7 days before discarded as negative.

Identification of isolated yeast

a. Macro morphology, conventional methods: Isolated colonies were described considering the size, consistency and pigmentation if present. Isolated *Trichosporon* characterized by being flat, creamy, moistened, wrinkled in the centre.

b. Microscopic examination by each of wet preparation and gram stained smears showing yeast cells: *Trichosporon* species are characterized by hyphae, and arthrospores which are gram positive in reaction.

c. Subculture on rice agar plates: 20 gm of rice were added to 400 ml water and boiled then simmered over a low flame for 45 min. The liquid was filtered and 20 gm of agar were added to the filtrate and completed to one liter of distilled water, then the mixture was autoclaved at 121°C for 15 min. The poured plates of rice agar were inoculated with yeast to be identified in short 3 to 4 streaks and covered with sterile cover slip, incubated at 25°C for 48 h, then examined microscopically for detection of blastospores, hyphae, pseudohyphae and arthrospores.

In this study, 14 isolates were identified phenotypically as *Trichosporon* species. They were subjected to PCR.

d. PCR: Genomic DNA of isolated yeast species was extracted.

DNA extraction was performed according to Sugita et al. (1998a). Briefly, one loop of yeast cells was suspended in lysing solution (100 mM Tris-HCl [pH 8.0], 30 mM EDTA, and 0.5% sodium dodecyl sulfate) and then heated at 100°C for 15 min. The solution was extracted with phenol-chloroform- isoamyl alcohol (25:24:1 [vol/vol]). DNA was precipitated with cold isopropanol.

Table 1. Trichosporon genus specific primers.

Primer	Primer design	
Forward	–	- .
Reverse	–	- .

PCR amplification

Three different PCR protocols were performed using 3 different primer pairs. The first primer pairs (Sugita et al., 1998a), would specifically amplify only *Trichosporon* species. They were chosen to align with regions on small subunit (SSU) of ribosomal DNA (rDNA) which were not conserved in other medically important yeasts. The amplification reactions were performed according to author's instructions (Sugita et al., 1998a).

Sixty- eight yeast isolates were subjected to PCR using *T. genus* specific primers. Positive bands detected at 170 bp were obtained in 16/68(23.5%) samples while the remaining 52(76.5%) yeast isolates were negative. The 14(16) positive PCR cases were previously identified phenotypically as *Trichosporon* species while the two cases were falsely diagnosed negative by conventional methods (Table 1).

The positively identified 16 isolates were subjected to subsequent two PCR runs using 2 different primer pairs that would specifically amplify *T. asahii* or *T. mucoides* DNA.

The variable interspacer region (ITS) located between 18 S and 26 S r RNA genes and comprising ITS-I (between 18 S and 5.8 S rRNA genes) and ITS-2 (between 5.8 S and 26 S rRNA), showing the location of the *T. asahii*- specific primers (TASF and TASR) and *T. mucoides* primers (TMF and TMR) (Ahmad et al., 2005). The amplification reactions were performed according to authors instructions (Ahmad et al., 2005; Sugita et al., 1998b) (Table 2).

RESULTS

There were 7(10.7%) yeast isolates out of 65 cases suffering from persistent fever on top of hematologic malignancies. 32(16.67%) yeast isolates were recovered out of 192 cases of onychomycosis, and 29(20.7%) out of 140 cases of *T. pedis*.

A total of 14(3.5%) isolates were identified by conventional methods as *Trichosporon* species. Phenotypic identification was based on macro morphology on SDA, gram stained smears, subculture and microscopic characters on rice agar plates. There were 0(0.0%), 9(4.68%) and 5(3.6%) isolates from cases with hematologic malignancies onchomycosis and *T. pedis*, respectively.

Performing PCR using *Trichosporon* genus specific primers for 68 yeast isolates revealed 16 positive samples. Two of them were negative by conventional methods. Subsequent PCR runs by specific primers revealed positive results in *T. asahii* for 13/16, while none of the samples was positive for *T. mucoides*.

Table 2. *T. asahii* and *T. mucoides* specific primers.

Species	Primer	Primer design	Amplicon size % (bp)
T. asahii	TASF	-- .	340
	TASR	-AGCA- .	
T. mucoides	TMF	--	416
	TMR	-- .	

Table 3. Frequency of isolated yeasts in the examined clinical samples.

Clinical cases	No of samples	No of isolated yeasts (%)
Hematological malignancies	65 blood cultures	7 (10.7)
Onychomycosis	192 nail scrapings	32 (16.6)
Tinea pedis	140 skin scrapings	29 (20.7)
Total	397	68 (17.1)

Table 4. Frequency of phenotypically identified *Trichosporon* in the examined specimens.

Cases	No of identified *Trichosporon*	
	No	%
Blood cultures (65)	0/65	0.0
Nail scrapings (192)	9/192	4.68
Skin scrapings (140)	5 /140	3.6
Total (397)	14/397	3.5

DISCUSSION

It is important to emphasize that emergent fungal infections are usually difficult to diagnose, refractory to conventional antifungal drugs and associated with high mortality rates (Colombo et al., 2003; Walsh et al., 2004). In patients with malignant hematological diseases, genus *Trichosporon* has been reported as the second most common agent of yeast disseminated infections; only behind the genus *Candida* (Fleming et al., 2002).

In the current study, yeast isolates were obtained from 68/397(17.1%) samples including 65 blood cultures, 192 nail scrapings, 140 skin scrapings. Yeasts were isolated from 10.7, 16.6 and 20.7% of cases with hematological malignancies, onychomycosis, and those with *T. pedis*, respectively. *Trichosporon* was identified from 9(4.68%) nail samples, 5(3.6%) skin samples and none of blood cultures based on the morphologic characters of isolated colonies on SDA, microscopic examination of colonies on rice agar (Tables 3 and 4).

Trichosporon species are mostly associated with benign superficial lesions (Gueho et al., 1994). Some Mexican authors have documented that the isolation of *Trichosporon* species from *T. pedis* and onchyomycosis ranged from 2.8 to 42.8% of cases (Mendez-Tovar et al., 2006).

In a study on the athlete's foot and onychomycosis by Archer-Dubon et al. (2003), they mentioned that the most frequently isolated fungus in their patients was *Trichosporon cutaneum*. This species is subdivided into *T. asahii*, *Trichosporon ovoides*, *Trichosporon inkin*, *T. mucoides*, *Trichosporon asteroids* and *T. cutaneus*.

T. asahii is the most clinical important pathogenic yeast in the genus *Trichosporon.* It causes onychomycosis, visceral infection, and summer- type hypersensitivity pneumonitis (Gueho et al., 1994). Generalized infections have been reported mostly in immunocompromised patients, including those with hematologic malignancies (Nakagawa et al., 2000).

Trichosporon species, for example *T. asahii* and *T. mucoides* have been described as emergent opportunistic pathogens related to disseminated infections in immunocompromised patients (Groll and Walsh, 2001). However, Rastogi and Nirwan (2007) reported a rare case of meningoencephalitis and pneumonia due to *T. asahii* in an immunocompetent patient. This fact demonstrates the pathogenic role of *Trichosporon* species to cause human diseases.

Several methods used for *Trichosporon* species identification have been reported, including morphological and biochemical tests and the use of molecular tools (Walsh et al., 2004) . Phenotypic methods are based on the characterization of micro morphological aspects of the colonies as well as the biochemical profiling. Performing a slide micro culture to search for arthroconidia is a very useful tool for identification. However, the accuracy of the phenotypic identification of *Trichosporon* species seems to be limited (Sugita et al., 1995). Evaluation of specific nucleotide sequences can be a precise method to resolve taxonomic and epidemiological problems generated by the phenotypic identification of *Trichosporon* species (Sugita et al., 1998a). Taj-Aldeen et al. (2009), stated that moleculer biologic technique (PCR) is more precise for *Trichosporon* identification.

In this study, PCR using *Trichosporon* genus specific primers for 68 yeast isolates was positive in 16 samples (14 were previously identified by morphology and 2 nail scrapings were falsely diagnosed negative by morphology) at 170 bp. It was stated by Sugita et al. (1998a), that to detect *Trichosporon species*, they designed genus specific primers for PCR, based on small subunit (SSU) rDNA sequences that produced a 170 bp fragment for positive samples. They concluded that the method presented in their study can specifically detect the DNA of *Trichosporon* species.

Molecular methods are more precise for identification but are still costly for routine laboratory (Sugita et al., 2002; Walsh et al., 2004) . In our study, we performed subsequently 2 PCR runs on the identified 16 samples using specific primers for each of *T. asahii* and *T. mucoides*. Their sequences included ITS1, ITS2. They yielded specific amplification of a DNA fragment at 430 bp in 13/16 samples, which is specific for *T. asahii* and 0(0.0%) sample was positive for *T. mucoides* specific primers.

Sugita et al. (1998a) constructed a phylogenetic tree with the small subunit (SSU) region sequences of rDNA for specific identification of the genus *Trichosporon*. Subsequently, Sugita et al. (1999) have sequenced and analyzed the interspacer regions (ITS-1 and ITS-2) genes of DNA from *T. species*. The ITS-1 and ITS-2 regions are highly conserved among *Trichosporon* spices and sequencing of those two regions is sufficient to establish the identity of *Trichosporon* or other fungal organisms (Taj-Aldeen et al., 2009).

Several methods used for *Trichosporon* species identification have been reported including morphological and biochemical tests and the use of molecular tools. Despite the fact that phenotypic methods are more suitable for routine in general microbiology laboratories, the accuracy for identification seems to be limited (Sugita et al., 1994; Walsh et al., 2004).

In our study, the amplification of DNA (TISI and ITS2) from 13(72.2%) of 16 clinical isolates with *T. asahii* specific primers, suggested that the 13 isolates were actually *T. asahii* strains, while the remaining 3 isolates remained unidentified.

Up to our best knowledge, it is the first time to isolate and identify *Trichosporon* species in Egypt. This study suggests that *T. asahii* is the most common species associated with human clinical trichosporonosis in our locality.

Conclusion

Occurance of trichosporonosis is not rare in human. *T. asahii* species is common in our locality. Molecular methods are more precise for identification of *Trichosporon*. Therefore, the standardization of laboratory methods for *Trichosporon* identification and antifungal susceptibility tests are necessary to investigate for both superficial and systemic trichosporonosis.

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