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Original Article

The evaluation of MALDI-TOF and conventional methods for the identification of *Trichosporon species* isolated from onychomycosis infection

Rabiye Altınbaş[1], Hafize Sav[2]

[1]*Dr. Ersin Arslan Training and Research Hospital, Department of Mycology, Gaziantep, Turkey*

[2]*Kayseri City Hospital, Department of Mycology, Kayseri, Turkey*

Abstract

Objective: Onychomycosis is one of the most common nail diseases and accounts for approximately half of all nail abnormalities. It has been noted that yeast-like microorganisms of the genus *Trichosporon*, which are the cause of uncommon but medically important infections, have increased in recent years as fungal agents causing onychomycosis. In this study, it was aimed to evaluate the MALDI-TOF MS and conventional methods used for identification of *Trichosporon* species isolated from nail samples.

Methods: The Cerrahpasa Medical Faculty Mycology Laboratory nail sample records of two years were retrospectively reviewed. The performance of conventional methods (morphological and biochemical identification by API 20C AUX), and MALDI-TOF MS method was compared for identification performance at the species level.

Results: The gender distribution of the patients with *Trichosporon* isolated samples was found as 67% female and 33% male. The anatomical region involvement was 97% toenail and 3% fingernail. Direct microscopy with KOH was found to be positive in 73% of nail samples. Identification performance at the species level of the MALDI-TOF MS method was found higher than the conventional method. 100% of the 33 non-*Candida* yeasts were defined as *Trichosporon* spp. with both methods. Among those, 58% were identified at the species level by conventional method and 85% by MALDI-TOF MS method. Non-*Candida* yeast distribution of 33 isolates by the API 20C AUX method was 40% *T. asahii*, 12% *T. mucoides*, 6% *T. inkin* and it was 40% *T. asahii*, 30% *T. mucoides*, 9% *T. inkin*, 6% *Trichosporon debeurmannianum* by the MALDI-TOF MS method.

Conclusion: The MALDI-TOF MS method was found to be superior to the conventional method in *Trichosporon* species identification.

Keywords: MALDI-TOF, onychomycosis, *Trichosporon species*, yeast identification

Address for correspondence: Rabiye Altınbaş, Dr. Ersin Arslan Training and Research Hospital Mücahitler, Gazi Muhtar Paşa Blv. No: 37, 27090, Gaziantep, Turkey

Phone: +90 532 704 49 67 **E-mail:** rabiaoguz@gmail.com **ORCID:** 0000-0003-2535-0480

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INTRODUCTION

Onychomycosis is one of the most common nail diseases and accounts for approximately half of all nail abnormalities. Dermatophytes are the most common cause of onychomycosis. Non-dermatophyte molds and yeasts also constitute about 10% of nail infections. Among yeasts, *Candida albicans* is the most common, responsible for candidial onychomycosis infections. It has been noted that yeast-like microorganisms of the genus *Trichosporon*, which are the cause of uncommon but medically important infections, have increased in recent years as fungal agents causing onychomycosis (1).

The *Trichosporon* species are found in phylum Basidiomycota (2). A recent taxonomi which used IGS1 rDNA sequence analysis, identified 20 species within the genus (3). Among these species, *T. asahii*, *T. asteroides*, *T. inkin*, *T. ovoides*, and *T. faecale* were reported as infectious in humans (4, 5, 6).

The most common form of superficial infection in humans is white piedra. Since different *Trichosporon* strains have resistance to some antifungals, so *Trichosporon* species identification is critical to correct treatment (7). Therefore, researches on the accurate identification, antifungal susceptibility patterns, and epidemiology of the species are very important. Along with the macroscopic and microscopic evaluation of colonies, commercial identification methods API ID 20C (bioMérieux, Marcy I'Etoile, France), and API ID 32C (bioMérieux, Marcy IEtoile, France), Phoenix (Becton Dickinson Diagnostics, Sparks, MD, USA), VITEK 2 (bioMérieux, Marcy I'Etoile, France) can be used in the laboratory for the identification of *Trichosporon* species, but the fact that the databases of these methods contain a limited number of *Trichosporon* species emerges as a situation that may limit their use. Besides, various molecular methods (ribosomal DNA sequence) are used in species identification (8), but it is not preferred to use molecular methods routinely due to the need for a laboratory with advanced types of equipment, high costs, experienced staff, and difficulties in applications (9). With the search for tests that give faster results instead of time-consuming and labor-intensive tests, the matrix-mediated laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) method has begun to be recommended as an alternative for routine use in microbiology laboratories for both bacteria and fungi identification (10). This method is easy to apply, providing the correct species identification within minutes (11).

Antifungal susceptibility profiles may vary among *Trichosporon* species so precise identification to the species level is crucial for better epidemiological understanding of this genus. Although *Trichosporon asahii* is the most common etiological agent of trichosporonosis, to the best of our knowledge, only limited data are available on the species distribution of *Trichosporon* species isolated from nail specimens. In this study, it was aimed to evaluate the MALDI-TOF MS and conventional methods used for identification of *Trichosporon* species isolated from nail samples.

MATERIALS AND METHODS

Patients

Our study included 251 patients who were admitted to the Cerrahpasa Medical Faculty Mycology Laboratory. First, the nails of the patients were wiped with 70% alcohol and they were allowed to dry. The nails were then cut with a sterile nail cutter to include as much of the proximal part of the lesion as possible.

Conventional method with API 20C AUX method (bioMérieux, France)

The nail parts taken to the slide were examined microscopically for fungal hyphae (true and pseudo hyphae) and spores (arthrospore, blastospore) with KOH solution (10-30%) (Figure 1) (12, 13). For culture, the samples were inoculated on SDA medium (HiMedia, India) with or without cycloheximide, and the culture plates were incubated at 25 °C and 35 °C. Cycloheximide tolerance of the species was examined. Colonies that grew at the end of the incubation were examined macroscopically and microscopically to diagnose the possible agent. On macroscopic examination,

Figure 1. Direct microscopical evaluation of KOH positive hyphae and spores

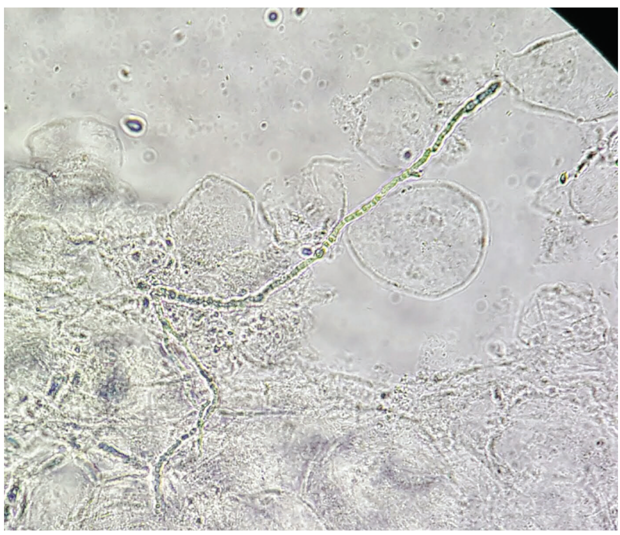

Figure 2. Sabouroud dextrose agar (SDA) medium-*Trichosporon spp.* colony (macroscopic)

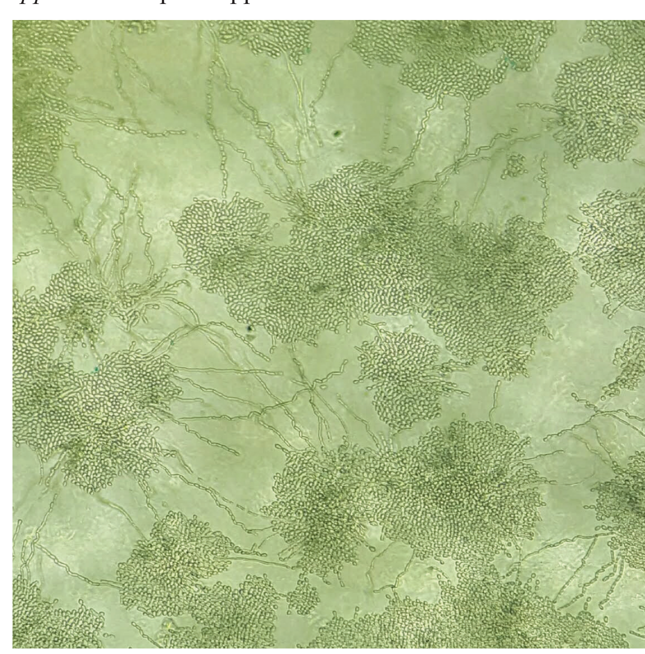

Figure 3. Corn meal-tween 80 agar medium-*Trichosporon spp.* microscopical appearance

while young colonies appeared smooth, old colonies showed grooves in the form of cerebral folds (Figure 2). In order to determine the morphological characteristics of yeasts and to contribute to species identification, corn meal / Tween-80 agar medium (HiMedia, India) was cultivated using the "Dalmau" method. The species were microscopically examined for the presence of true hyphae, pseudo-hyphae, numerous unicellular blastospore, and cubical, barrel or elongated arthrospore (Figure 3). The dark blue color formed in chromogenic agar medium (HiMedia, India) and urease enzyme activities in Christensen's urea agar medium (HiMedia, India) were investigated. Besides, species identification was made from breeding fungal colonies with API 20C AUX (bioMérieux, France) Commercial Kit according to carbohydrate and nitrogen assimilation properties.

MALDI-TOF MS (Bruker Daltonics, Germany) method:

Protein extraction from yeasts was performed by the ethanol / formic acid method in line with the manufacturer's recommendations. 1 µl of the supernatant formed after extraction was taken and placed in two separate points on the plate. Immediately after the liquid at these points dried, 1 µl of matrix solution (α-cyano-4-hydroxycinnamic acid) was added to the sample. After drying, the plate was placed in MALDI-TOF MS (Bruker Daltonics, Germany) and protein profiles and their mass spectrometers were obtained. The data obtained were evaluated using the database of the device and yeast isolates were identified. In line with the manufacturer's recommendations, results with score values ≥ 2.0 were considered significant at the species level, and results between 1.7-1.99 were considered reliable at the genus level. Results with score values <1.7 were considered unreliable (8).

Table 1. Direct microscopical evaluation of 33 *Trichosporon species* growing in culture

	KOH positive	KOH negative
***Trichosporon* spp. (n=5)**	5	-
***Trichosporon asahii* (n=13)**	8	5
***Trichosporon mucoides* (n=10)**	7	3
***Trichosporon inkin* (n=3)**	2	1
***Trichosporon debeurmannianum* (n=2)**	2	-
Total (n=33)	**72.7%** (24/33)	**27.3** (9/33)

RESULTS

The gender distribution of the patients with *Trichosporon* isolated samples was

Table 2. Distribution of *Trichosporon* isolates according to API 20C AUX and MALDI-TOF MS methods

	API 20C AUX		MALDI-TOF MS	
	Number	%	Number	%
Trichosporon sp.	14	42	5	15
Trichosporon asahii	13	40	13	40
Trichosporon mucoides	4	12	10	30
Trichosporon inkin	2	6	3	9
Trichosporon debeurmannianum	0	0	2	6
Total	33	100	33	100

found as 67% female and 33% male, and the anatomical region involvement was 97% (32/33) toenail and 3% (1/33) fingernail. Direct microscopy with KOH was found to be positive in 24 (73%) of 33 nail samples that grew *Trichosporon* spp. in culture, and negative in the remaining 9 samples (27%) (**Table 1**).

Distribution of 33 isolates defined as non-*Candida* yeast by the API 20C AUX method was 13 (40%) *T. asahii*, 4 (12%) *T. mucoides*, and 2 (6%) *T. inkin*. Species-level identification of 14 (42%) isolates was not successful with this method; they were described as *Trichosporon* spp.

Distribution of 33 isolates identified as non-candida yeast according to MALDI-TOF MS method was 13 (40%) *T. asahii*, 10 (30%) *T. mucoides*, 3 (9%) *T. inkin*, and 2 (6%) *Trichosporon debeurmannianum* (**Table 2**). Species-level identification of 5 (15%) isolates was not successful with this method; they were described as *Trichosporon* spp. *Trichosporon asahii* was the most frequently isolated species according to both methods, followed by *T. mucoides* and *T. inkin*. 13 *T. asahii* isolates identified by the API 20C AUX method were also identified as *T. asahii* with MALDI-TOF. In addition to 4 *T. mucoides* species identified by the API 20C AUX method, 6 more *T. mucoides* species were identified with MALDI-TOF MS. Besides, unlike the API 20C AUX method, 2 *T. debeurmannianum* species were identified with the MALDI-TOF MS method. The score values of all microorganisms identified with MALDI-TOF MS were found to be ≥ 2.0 and these results were considered to be correctly identified. According to these results, 100% of the 33 non-*Candida* yeasts were defined as *Trichosporon* spp. with both methods. Among those, 58% were identified at the species level by API 20C AUX method and 85% by MALDI-TOF MS method. Identification performance at the species level of the MALDI-TOF MS method was found higher than the API 20C AUX method.

DISCUSSION

Low socio-economic condition, nail trauma, poor hygiene, and climatic conditions were important of the predisposing factors observed of nail fungal infections. It can be caused by dermatophytes, yeasts and non-dermatophyte molds.

Trichosporon species occur as a natural part of the human skin microbiota. However, they are opportunistic and considered fungal pathogens that occur in immunocompromised hosts (14). Recently, the increase in the frequency of onychomycosis caused by *Trichosporon* species has been striking (9). *Trichosporon cutaneum* has been reported to be the most common yeast in onychomycosis (15).

Trichosporon species are reported as agents with varying rates of 0.1–35.5% in patients with onychomycosis. In particular, the frequency of *Trichosporon* isolation from onychomycosis in Turkey (9.49%), Korea (10.1%), and Nigeria (10.3%) is

Table 3. Distribution of superficial mycology samples

Samples		Number	%
Nail	Toe nail	177	70.52
	Hand nail	30	11.95
Others (Skin scrapings, hair, scalp)		44	17.53
Total		251	100.00

quite high (16, 17). Han et al from Korea found the prevalence of *T. cutaneum* as 10.1% in 2591 nail samples they examined in a study they conducted. Moreover, *T. cutaneum* is stated to be the second most frequently isolated fungus after *T. rubrum* (18). Gündüz et al., in their study to investigate the frequency and distribution of onychomycosis in school children, found that there was culture growth at a rate of 0.1%. The most common agents in culture were *Trichosporon* spp. (45.8%) and *Trichophyton rubrum* (25%) (19). In two articles with 467 and 98 onychomycosis cases from Mexico *Trichosporon* spp. found in 35.5% and 18.3% of the patients, respectively (20, 21). In a multicenter study involving 2731 patients with positive onychomycosis culture in Argentina, only 8 (0.3%) cases were found with *Trichosporon* spp. growth (22).

Although *Trichosporon* species have rarely been implicated as causative agents of onychomycosis, they are also emerging as important etiological agents of onychomycosis (7). Among the isolated species of *Trichosporon*, *T. asahii* was recovered from the nail samples of all the patients, its recognition as a main pathogenic species of onychomycosis (23).

In this study, we analyzed 33 clinical *Trichosporon* isolates. The isolates were identified as belonging to the *Trichosporon* genus by using both API 20C AUX method, including morphological characteristics and microscopic properties on cornmeal Tween 80, and MALDI-TOF MS method. The isolates represented four species with a MALDI-TOF MS method and three species with a API 20C AUX method. Culture-based phenotypic methods, such as the commercial API 20 C AUX, usually generate inconclusive results compared to MALDI-TOF MS method (24).

In our study, culture samples were taken from 251 patients with suspected fungal infection and 33 *Trichosporon* spp. were isolated. Candidal onychomycosis mainly affects fingernails although *Trichosporons* are yeast-like fungi, all isolates except one (97%) affected the toenails in our study by consistent with the literature (23). The distribution of isolated samples by patient gender was higher in female (67%) than in male (33%). These results are in contrast with many of the studies in the worldwide literature (25, 26).

In direct microscopic evaluation, which provides an important guide for fungal infection of the nail and is a valuable finding, the positivity rate is stated to be 60-80%. However, the positive direct microscopic result was found to be 0.18% in the study conducted by Gündüz et al. to investigate the frequency and distribution of onychomycosis in school children (19). In 24 (73%) of the 33 *Trichosporon* isolates included in our study, fungal hyphae and/or spores were seen in direct microscopy with KOH and direct microscopy was evaluated as positive.

Defining *Trichosporon* isolates not only at the genus but also at the species level is very important in guiding the antifungal treatment because the species show different resistance profiles. For example, in vitro, *T. asahii* isolates are more resistant to amphotericin B (AMB) compared to triazole agents, and *Trichosporon* species other than *T. asahii* are more resistant to triazole derivatives compared to AMB. In addition, if *Trichosporon* species are treated with echinocandins, breakthrough infections may be seen due to intrinsic resistance, and voriconazole is preferred as a primary antifungal in invasive *Trichosporon* infections, the correct and rapid definition of *Trichosporon* is a great importance isolated from clinical samples (27). For this reason, species identification is given importance and new commercial identification kits are developed. MALDI-TOF MS, which is used to identify microorganisms in the microbiology laboratory, stands out as an alternative method due to its reliable results, fast and easy application, and low cost (4, 28). Kolecka et al. in their studies, correctly identified 41 (91.1%) of 45 culture collection isolates and 101 (99.0%) of 102 isolates of 10 different *Trichosporon* species isolated from clinical samples with the Bruker MALDI-TOF MS method. However, the authors emphasized that very similar species *T. inkin / T. ovoides*, *T. japonicum / T. asteroids*, and *T. dermatis / T.* mucoides could not be defined correctly (8). In the study comparing time and cost with API 20 C AUX yeast identification methods, Bruker Biotyper MALDI-TOF MS method in the identification of 103 rare yeasts including 3 *T. asahii* and 3 *T. mucoides*, was found to be superior in terms of cost and time (29).

Limitations:

The main limitation of our study is that 33 *Trichosporon* spp isolates could not be identified by the sequence method. Identification by the molecular method cannot be used routinely in terms of both cost and qualified

personnel. According to the data of our study, it was found that only 5 *Trichosporon* spp. isolates could not be identified at the subspecies level in the identification of 33 *Trichosporon* spp. isolates with the MALDI-TOF MS method. Subspecies of 14 *Trichosporon* spp. isolates could not be identified by the conventional method. The agreement between the two methods in identifying the *T. asahii* species was 100%. It is thought that the presence of *T. asahii* species in the database of the API 20 C AUX method used in conventional identification may contribute to this method.

CONCLUSION

Trichosporon spp is a cause of deep and superficial mycosis. Species identification is important for appropriate antifungal selection in the treatment of fungal infections caused by these factors. When the identification of *Trichosporon* spp. isolates was compared with the two methods, the MALDI-TOF MS method was found to be superior to the conventional method in *Trichosporon* species identification. Although conventional methods based on morphological and biochemical properties are suitable for routine use in medical microbiology laboratories, they remain limited to identify *Trichosporon* spp.

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Author contributions: Design of the study; RA - Supervision; RA, HS - Data collection &/or processing; RA, HS - Performed data analysis; RA - Literature search; RA, HS - Written by; RA - Critical review; RA, HS

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