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Evaluation of the MALDI-TOF MS method for routine identification of *Candida* species in a tertiary care hospital in Thailand

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ABSTRACT

Aims: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is increasingly used to identify *Candida* spp. in diagnostic laboratories due to its strength in providing accurate information results, speed and cost-effectiveness. However, its accuracy varies on instrument platform, reference database, sample preparation techniques and interlaboratory comparisons. Therefore, the use of MALDI-TOF MS for species identification was evaluated against traditional biochemical identification, namely Brilliance^{TM} *Candida* and Remel RapID^{TM} Yeast Plus System.
Methodology and results: To evaluate and compare identification efficiency, turnaround time and consumable cost, 194 clinical isolates of *Candida* were collected. The results showed overall 85.6% concordant identification between two methods with 94.9-99.5% and 100% accuracy in traditional and MALDI-TOF MS, respectively, in the identification of four common *Candida* species; *C. albicans*, *C. tropicalis*, *C. parapsilosis* sensu stricto and *C. glabrata* sensu stricto. Other *Candida* species were also identified with 85.6% and 97.5% accuracy rates by traditional and MALDI-TOF MS, respectively. Additionally, identification using MALDI-TOF MS reduced overall turnaround time and cost by approximately 99.8% and 86.5%, respectively.
Conclusion, significance and impact of study: This study highlights the performance of MALDI-TOF MS, which is more accurate in identifying *Candida* spp. with a less hands-on approach, cheaper cost and shorter turnaround time.

Keywords: MALDI-TOF MS, identification, *Candida*, clinical diagnosis

INTRODUCTION

The incidence of invasive fungal infections has increased significantly in immunocompromised patients, especially those requiring long-term catheterization, organ transplants or immunosuppressive drugs. The most prevalent cause of fungal infections is the *Candida* species, followed by the *Aspergillus* species (Badiee and Hashemizadeh, 2014). Among *Candida* species, *C. albicans* remains the most frequently isolated in immunocompromised patients. However, in the last decade, the prevalence of non-*albicans Candida* species, including *C. tropicalis*, *C. parapsilosis* sensu stricto and *C. glabrata* sensu stricto has increased worldwide (Guinea, 2014). In Thailand, the epidemiological trend has also shifted from *C. albicans* towards non-*albicans Candida*, which has reduced susceptibility to antifungal drugs (Tritipwanit *et al.*, 2005; Chaiwarith *et al.*, 2011).

To identify fungal pathogens, clinical laboratories often use traditional methods that rely on macroscopic and microscopic morphologies and biochemical properties of causative agents (Chindamporn *et al.*, 2018; Wang *et al.*, 2020). However, these traditional methods are time-consuming (2-5 days) as microorganism growth is required to define species based on their physiological properties and growth characteristics. Therefore, a multipurpose culture medium that can support growth, inhibit contaminants and differentiate between species is commercially available. Recently, a chromogenic medium for *Candida* or Brilliance^{TM} *Candida* agar has been developed. This medium facilitates yeast isolation and can differentiate between three *Candida* species, including *C. albicans*, *C. tropicalis* and *Issatchenkia orientalis*. These three *Candida* species exhibit different patterns in hexosaminidase and alkaline phosphatase production due to two specific chromogenic substrates in the medium, which are cleaved by the enzyme produced by yeast, resulting in different colony colors. Although this chromogenic formulation can identify species of *Candida* within 48 h, the result is limited to only these three

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Candida species. The identification of clinically important *Candida* species other than *C. albicans*, *C. tropicalis* and *I. orientalis* is still needed.

In addition, rapid biochemical tests have also been developed to identify a broader range of *Candida* spp. One of these is a commercial yeast identification kit known as the Remel RapID™ Yeast Plus System or API20C AUX. With five-carbon source assimilation tests and thirteen enzymatic hydrolysis substrates, this test can identify 19 species of *Candida* (Smith *et al.*, 1999). This test also helps to decrease turnaround time since no additional tests are required, but it still requires an incubation period of up to 72 h and the results are often difficult to interpret. However, the Remel RapID™ Yeast Plus System cannot differentiate between closely related species. For instance, *C. dubliniensis* and *C. albicans* are two pathogenic species that share several phenotypic properties and often difficult to tell apart (Ells *et al.*, 2011). Also, morphological and biochemical properties alone cannot help distinguishing complex species like *C. parapsilosis* sensu stricto/*C. metapsilosis*/*C. orthopsilosis*.

While the molecular method remains the gold standard or reference method for microbial identification, it requires expensive equipment and expertise, has a turnaround time of several days, is cost-ineffective and is not easily applicable in a routine diagnostic laboratory in Thailand (Chindamporn *et al.*, 2018). However, in the past decade, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been developed to identify various species through specific protein profiling of microorganisms. Moreover, MALDI-TOF MS has also been implemented in the field of clinical diagnostic microbiology. This technique has been widely adopted due to its advantages of being rapid, reliable, easy to use, cost-effective and the possibility to test for a wide variety and number of organisms (Singhal *et al.*, 2015). Furthermore, MALDI-TOF MS has been shown to correctly identify or detect microorganisms in clinical specimens, i.e., blood culture (Spanu *et al.*, 2012) and urine. Also, it dramatically reduces turnaround time which makes it easy to manage effective therapies.

To help identify species of clinically relevant yeast samples, MALDI-TOF MS is increasingly replacing biochemical differentiation approaches. MALDI-TOF MS can identify a yeast colony grown in a culture medium within minutes and the results are highly accurate and reliable (van Belkum *et al.*, 2015). However, misidentification of species using the MALDI-TOF MS has also been reported (Dhiman *et al.*, 2011; Won *et al.*, 2013). Several studies have shown that misidentification and a failure to report the correct species vary based on reference database (Mancini *et al.*, 2013), the machine platform (Rosenvinge *et al.*, 2013), sample preparation method (Faron *et al.*, 2015) and interlaboratory comparisons (Won *et al.*, 2013). Thus, minor discrepancies in results acquired from traditional, biochemical, molecular and MALDI-TOF MS tests should be carefully observed. Also, the investment cost for implementing a new platform like the MALDI-TOF MS into the laboratory is a significant factor to consider as well.

In this study, we compared the efficacy of species identification between the traditional method and MALDI-TOF MS using nucleotide sequencing as the standard method. Furthermore, the comparison of these two approaches in terms of turnaround time and cost-effectiveness is also demonstrated.

MATERIALS AND METHODS

Isolates used in this study

In September 2019, a total of 194 clinical isolates of *Candida* spp. were collected from various specimens, i.e., hemoculture, body fluid (Jackson-Pratt drain), tissue biopsy (duodenum, psoas and axilla), percutaneous drainage pus, bronchoalveolar lavage, and wash, sputum, swab (cervical, vagina, skin, throat and oral), urine and feces. These specimens were sent for culture at the Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University.

Traditional identification

All clinical isolates of *Candida* spp. were identified by subculture on Brilliance™ *Candida* agar (Oxoid, Hampshire, United Kingdom) according to the manufacturer's instruction. After 24 h of incubation at 37 °C, *C. albicans*, *C. tropicalis* and *I. orientalis* were differentiated based on the color of their colonies. Meanwhile, the remaining *Candida* species were identified using the Remel RapID™ Yeast Plus System (Remel Inc., Lenexa, KS) following the manufacturer's recommendations.

Species identification by MALDI-TOF MS

Sample preparations of MALDI-TOF MS were made using the extended direct transfer method following the manufacturer's instructions (Bruker Daltonics, Bremen, Germany). First, a fresh colony grown on Sabouraud dextrose agar (SDA; Oxoid, Hampshire, United Kingdom) was directly spotted as a thin film onto a ground steel target plate in duplication. Next, 1 µL of 70% formic acid was added to a sample spot and allowed to air- dry. Then, the air-dried spot was overlaid with 1 µL of a saturated α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution in 50% acetonitrile and 2.5% trifluoroacetic acid and allowed to air-dry again. Subsequently, the target plate was analyzed by a Microflex LT mass spectrometer (Bruker Daltonics, Bremen, Germany) which was equipped with MALDI Biotyper 3.0 software containing 5627 main spectrum profiles (MSPs) on a reference database. According to the manufacturer's guidance, isolates with an identification score of ≥2.0 were marked as species-level identification, while isolates with a score <2.0 but ≥1.7 were marked as genus-level identification. However, a score below 1.7 was considered an unreliable identification.

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If an identification score was below 1.7, the isolate was tested further using the extraction method as per the manufacturer's instruction. The process began with one fresh colony cultured on SDA, which was then transferred and resuspended in 300 µL of HPLC-grade water. Next, 900 µL of absolute ethanol was added and thoroughly mixed. The mixture was then centrifuged for two min at 14,000 rpm, after which the supernatant was removed, and the pellet was allowed to air-dry for at least 5 min at 25-27 °C. After this step, 25 µL of 70% formic acid was added and the solution was resuspended until the pellet was completely dissolved. Next, 25 µL of acetonitrile was added and centrifuged for 2 min at 14,000 rpm. Subsequently, 1 µL of supernatant was smeared on the spot on the target plate in duplication and allowed to air-dry. Finally, the air-dried spot was overlaid with 1 µL of an HCCA matrix solution and subject to MALDI-TOF MS.

Nucleotide sequencing of internal transcribed spacer 1 and 2 (ITS1-ITS2) regions

To identify fungal species, ITS1-ITS2 sequencing was used as the gold standard. Genomic DNA was extracted from yeast colonies which were cultured on SDA for 24-48 h at 30 °C using the boiling method. First, a loopful of yeast cells was resuspended in 50 µL distilled water before being allowed to boil at 95 °C for 30 min and centrifuged at 10,000 rpm for 5 min. The DNA in the resulting supernatant was used as the template for PCR amplification of ITS1-ITS2 regions by using the ITS 1 (5' TCC GTA GGT GAA CCT GCG G 3') and ITS 4 (5' TCC TCC GCT TAT TGA TAT GC 3') primers as previously described (Pharkjaksu *et al.*, 2018). After the amplification, the PCR product was purified by a GeneJET^{TM} PCR Purification kit (Thermo Scientific, Lithuania) according to the manufacturer's recommendation. Then, the products were directly sequenced by Macrogen Inc., Korea, using 23 ABI 3730XLs DNA sequencing technology (Applied Biosystem, Foster City, USA). The ITS nucleotide sequences were then analyzed thoroughly using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) provided by the National Center for Biotechnology Information (NCBI) and International Society for Human and Animal Mycology (ISHAM) Barcoding database (http://its.mycologylab.org/).

Statistical comparison

Species identification by MALDI-TOF MS was compared to that done by the traditional method (culturing on Brilliance^{TM} *Candida* agar and biochemical testing by Remel RapID^{TM} Yeast Plus System for *Candida* species other than *C. albicans*, *C. tropicalis* and *I. orientalis*). The tests' sensitivity, specificity, accuracy, turnaround time and cost estimations were all assessed to ascertain their potential for identification using PASW Statistics for Windows (SPSS Inc., Chicago, IL, United States).

RESULTS

A total of 194 clinical isolates of yeasts were identified by the traditional method (culturing on Brilliance^{TM} *Candida* agar and biochemical testing by Remel RapID^{TM} Yeast Plus System for *Candida* species other than *C. albicans*, *C. tropicalis* and *I. orientalis*) and MALDI-TOF MS using ITS sequencing as the reference method. An ITS1-ITS2 sequence analysis identified 19 different species in the isolates: *C. albicans*, *C. tropicalis*, *C. parapsilosis* sensu stricto, *C. glabrata* sensu stricto, *I. orientalis*, *C. dubliniensis*, *C. orthopsilosis*, *C. lusitaniae*, *C. auris*, *C. duobushaemulonii*, *C. guilliermondii*, *C. haemulonii*, *C. nivariensis*, *C. pelliculosa*, *C. rugosa*, *C. diversa*, *C. fermentati*, *C. hellenica* and *C. vulturna* (Table 1). Most isolates (166/194; 85.6%) were correctly identified by both the traditional method and MALDI-TOF MS. Of the remaining 28 isolates, 24/194 (12.4%) were identified correctly by the MALDI-TOF MS method, while 4/194 (2.06%) could not be identified by either the traditional method or MALDI-TOF MS.

MALDI-TOF MS had an accuracy rate of 100% in all measured parameters among the four most frequently found species, namely *C. albicans*, *C. tropicalis*, *C. parapsilosis* sensu stricto and *C. glabrata* sensu stricto. Regarding species identification performance, the traditional method had less precision concerning specificity, accuracy and positive predictive value (PPV) (Table 2). The MALDI-TOF MS, on the other hand, had a greater sensitivity, accuracy and negative predictive value (NPV) in the identification of the other *Candida* species.

In terms of turnaround time and cost estimations, *C. albicans*, *C. tropicalis* and *I. orientalis* were grouped as they can be identified easily using Brilliance^{TM} *Candida* agar, however, the other *Candida* species required additional testing using the Remel RapID^{TM} Yeast Plus System. MALDI-TOF MS has a 99.8% lower turnaround time and a 71.4% higher cost estimate than the traditional method for these three species (Table 3).

DISCUSSION

An increase in the incidence of both common and rare species of fungal causative agents has emphasized the importance of rapid and accurate identification of fungal diseases. Compared with the traditional method, this study demonstrated that identifying species through pathogenic yeasts using MALDI-TOF MS could provide high levels of accuracy while substantially reducing turnaround time and testing costs.

MALDI-TOF MS was able to identify a wide variety of *Candida* spp. (190/194; 97.9%) of tested isolates while the traditional method achieved (166/194; 85.6%) identification accuracy. However, it was limited to just five species that are commonly isolated routinely in clinical laboratories. As the MALDI Biotyper software (version 3.0) contains 5627 MSPs including 22 species of *Candida*, several uncommon species such as *C. auris*, *C. lusitaniae*, *C. duobushaemulonii*, *C. guilliermondii*, *C. haemulonii*, *C. nivariensis*, *C. pelliculosa*, *C. rugosa*, *C.*

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Table 1: Comparison of the percentage of correct identification between the traditional method and MALDI-TOF MS.

Group[1)]	Species (number of isolates)	% of species identified correctly	
		Traditional method	MALDI-TOF MS
A	*C. albicans* (87)	100	100
	C. tropicalis (46)	100	100
	C. parapsilosis sensu stricto (18)	100	100
	C. glabrata sensu stricto (12)	100	100
	Issatchenkia orientalis (3)	100	100
B	*C. dubliniensis* (9)	0	100
	C. orthopsilosis (6)	0	100
	C. lusitaniae (2)	0	100
	C. auris (1)	0	100
	C. duobushaemulonii (1)	0	100
	C. guilliermondii (1)	0	100
	C. haemulonii (1)	0	100
	C. nivariensis (1)	0	100
	C. pelliculosa (1)	0	100
	C. rugosa (1)	0	100
C	*C. diversa* (1)	0	0
	C. fermentati (1)	0	0
	C. hellenica (1)	0	0
	C. vulturna (1)	0	0

Note: Groups[1)] were classified based on an agreement between the identification result by the traditional method and MALDI-TOF MS; Group A: Species identified correctly by both methods; Group B: Species identified correctly only by MALDI-TOF MS; Group C: Unable to be identified by either the traditional method or MALDI-TOF MS.

Table 2: Comparison of sensitivity specificity, accuracy, positive predictive value and negative predictive value between the traditional method and MALDI-TOF MS.

Organisms (number of isolates)	Results in the percentages of traditional method/MALDI-TOF MS				
	Sensitivity	Specificity	Accuracy	Positive predictive value	Negative predictive value
C. albicans (87)	100/100	90.7/100	94.9/100	89.7/100	100/100
C. tropicalis (46)	100/100	98.7/100	99.0/100	95.8/100	100/100
C. parapsilosis sensu stricto (18)	100/100	94.9/100	95.4/100	66.7/100	100/100
C. glabrata sensu stricto (12)	100/100	99.5/100	99.5/100	92.3/100	100/100
Other *Candida* spp. (31)	9.7/87.1	100/100	85.6/97.5	100/100	85.3/97.6

Table 3: Turnaround time and cost estimate per specimen between the traditional method and MALDI-TOF MS.

Identification method (number of isolates)	Results of traditional method/MALDI-TOF MS	
	Turnaround time (hours)	Cost per specimen (US dollars)
C. albicans, *C. tropicalis* and *I. orientalis* (136)	24.02/0.042	0.21/0.37
Other *Candida* spp. (58)	28.07/0.042	8.58/0.37

carpophila and *C. pseudohaemulonii* were correctly identified.

In addition, the test included species such as *C. albicans*/*C. dubliniensis*, *C. parapsilosis* sensu stricto/*C. orthopsilosis* which is closely related. In a general diagnostic microbiology laboratory, testing often fails to differentiate between these species, however, MALDI-TOF MS is able to discern each species correctly without requiring an additional step. For example, MALDI-TOF MS can correctly differentiate *C. albicans* from *C. dubliniensis* by using the typical data analysis workflow. While using the Brilliance^{TM} *Candida* agar solely, *C. dubliniensis* was misidentified as *C. albicans* because both species can produce hexosaminidase resulting in the same turquoise green color. To distinguish *C. dubliniensis* from *C. albicans*, several phenotypic and genotypic tests are needed (Neppelenbroek *et al.*, 2014; Jan *et al.*, 2017; Asadzadeh *et al.*, 2018) as illustrated in Figure 1. However, these tests are time-consuming and identification results remain unreliable. A comparison of *C. albicans* with *C. dubliniensis* shows that the latter is considerably less prevalent and virulent (Moran *et al.*, 2012). However, *C. dubliniensis* has become more resistant to fluconazole while *C. albicans* can be

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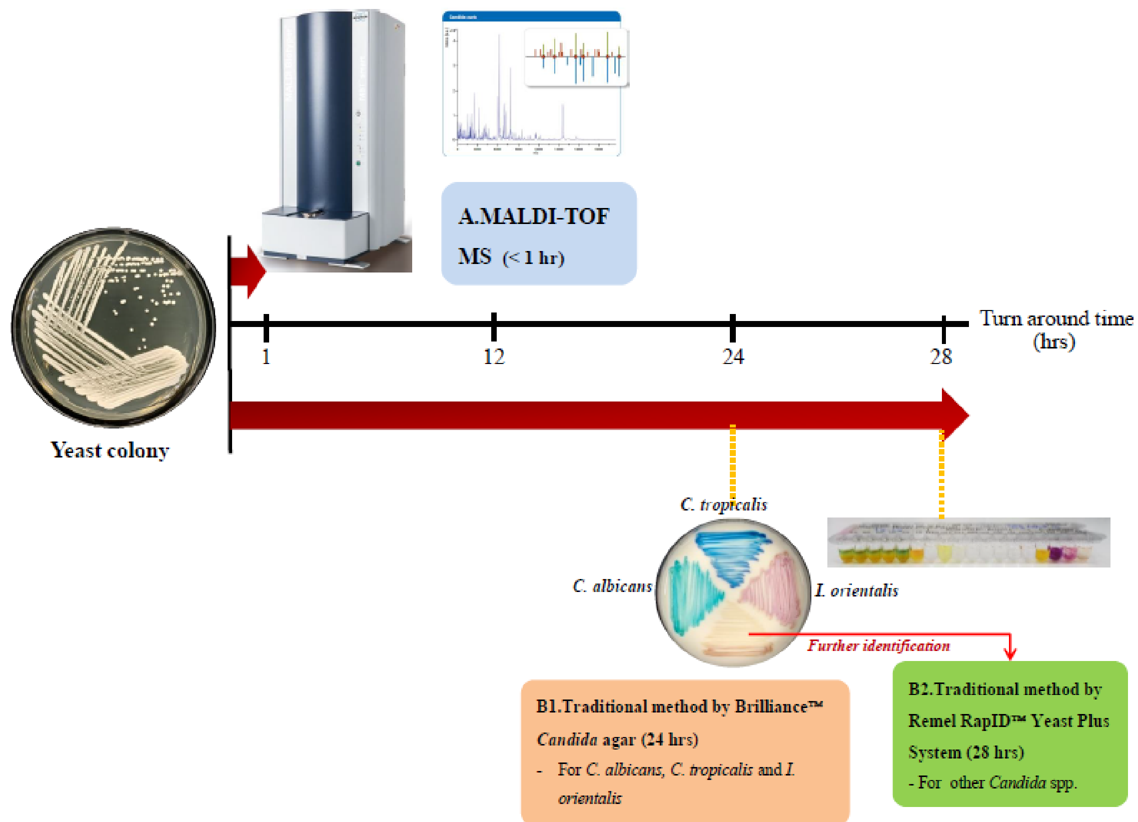

Figure 1: Comparison of identification workflow between (A) Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and (B1 and B2) Taditional method.

successfully treated (Moran *et al*., 1997; Pham *et al*., 2019). Errors in reporting a resistant species constitute a significant problem because the patient will not receive adequate treatment, leading to treatment failure. Similarly, *C. orthopsilosis* and *C. metapsilosis* have been noted to be more resistant to fluconazole than *C. parapsilosis* sensu stricto (Chen *et al*., 2010). Unfortunately, data regarding this complex species is still limited.

On the other hand, four isolates (2.06%) that are infrequently encountered in clinical specimens were unable to be accurately identified using MALDI-TOF MS due to a lack of reference spectra in the experiment's current database. Although the manufacturer keeps updating the database to increase the range of readily identifiable yeast, the latest version of the manufacturer-provided library (November 2020), which contains 9,607 MSPs including 73 *Candida* spp., still does not include those rare species found in this study. Those isolates rarely cause human infection and are uncommonly isolated from clinical samples (Brandt *et al*., 2004; Sengupta *et al*., 2013). However, many findings have revealed that the construction of a user-developed database enhances the ability of the MALDI-TOF MS to identify these hidden fungal species (Normand *et al*., 2013; Normand *et al*., 2017; Ceballos-Garzon *et al*., 2020). Besides just *Candida* spp., several other genera of filamentous fungi such as *Aspergillus* spp., *Fusarium* spp., *Scedosporium* spp. and Mucorales have also been successfully identified using an in-house database (Clark *et al*., 2013; Normand *et al*., 2013; Zvezdanova *et al*., 2019).

Apart from accurate and reliable identification, another major advantage of using the MALDI-TOF MS to define species is the substantially decreased turnaround time. Our findings found similar results as in previous studies (Dhiman *et al*., 2011; Sow *et al*., 2015). However, reduced turnaround time varies a little depending on the type of causative agents, extraction protocol, instrument platform and compared methods used. For example, causative agents that contain a thick cell wall might require a more complicated protocol, which will take a longer time to complete. The time saved allows for early diagnosis of yeast infections to help clinicians ensure rapid and efficient empirical treatments. As a result, this reduces the occurrence of drug-resistant pathogens, the mortality rate and the duration of hospitalization, particularly for severe and life-threatening infections.

In terms of price, the implementation of MALDI-TOF MS reduced the cost of consumable supplies and reagents by 95.7% compared to the traditional technique. Although the estimated cost was still more than when the only chromogenic medium was used, it is worth spending

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an additional 0.15 US dollars per sample for the potential of accurate identification and reducing hands-on time. This calculation is based on the testing of 20 samples of each batch which led to the determination of expected turnaround time and workload. However, costs can be minimized by increasing the sample number per batch to a maximum of 95 samples. This philosophy should be compromised with labor-intensive and hands-on time. Another point of contention was that this analysis omitted the initial instrumentation and maintenance costs, which may account for most of the cost of introducing a new technique. This, however, was attributable to the equipment being available in our institute since 2012. There is a model study that thoroughly calculates the required net cost (Rosenvinge *et al.*, 2013). It shows that the implementation of MALDI-TOF MS for routine identification saves nearly 90% of reagent costs and 50% of net cost.

The identification performance, cost-effectiveness and reduced turnaround time by implementation of MALDI-TOF MS may vary due to several factors. Firstly, the expertise of the technician is critical as MALDI-TOF identification results rely mainly on the number of growth colonies taken and smeared on the target plate. Secondly, routine maintenance, particularly laser calibration, is critical to preserving and improving the quality of results (Robert *et al.*, 2021). These high preventive maintenance costs substantially reduced cost-effectiveness. Thirdly, the total cost- and time-savings also vary among techniques used in each laboratory. Lastly, the time spent on each sample can be adjusted further during batch processing. However, MALDI-TOF MS is still strongly recommended in a hospital setting for routine identification of high volumes of samples.

This study assessed the performance of MALDI-TOF MS for species identification of *Candida* spp. A further study for the identification of *Cryptococcus neoformans*/*gattii* species complex is also warranted. Due to their thick capsule and cell wall, culture condition and protein extraction before analysis remain challenging processes (Thomaz *et al.*, 2016). In addition, filamentous fungi (such as *Aspergillus* spp.) require additional treatments with chemical reagents along with beating bead or heat to disrupt their strong cell walls for protein extraction (Cassagne *et al.*, 2011).

Strains recovered from clinical specimens might result in mixed cultures which had been previously reported as an obstacle for identification by MALDI-TOF MS (Schubert *et al.*, 2011). To address this issue, successful identification of mixed cultures at the species level was achieved utilizing MALDI-TOF MS and mixed culture algorithm software (Mörtelmaier *et al.*, 2019). However, the effectiveness of MALDI-TOF MS for mixed culture identification remains dependent on the target species and the fraction of species present in the mixed culture. Additionally, new elements are being developed to aid in the identification process when specimens, particularly positive hemocultures, are obtained directly. Unfortunately, the separation of causative agents directly from specimens and protein preparation to obtain a reliable result remains complex and requires an additional procedure. Nonetheless, the detection of antifungal resistance is fascinating. While there was a study on the successful detection of triazole resistance among *Candida* spp., improvements in reproducibility are still needed (Saracli *et al.*, 2015).

CONCLUSION

Our study revealed that the MALDI-TOF MS should replace the traditional technique due to its accuracy and potential for correctly identifying species. Subsequently, the use of MALDI-TOF MS reduces the amount of time necessary for testing and enhances its cost-effectiveness, particularly in a big hospital setting where a high number of samples are tested per batch. Uptake of this new technology will provide the benefits of early diagnosis and effective treatment.

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