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Application of Matrix-Assisted Laser Desorption Ionization in microbiological applications

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Abstract: Mass spectrometry became more and more widespread and many new ionization and ion separation techniques were introduced. For instance, matrix-assisted laser desorption ionization time-of-flight mass spectrometry is among the omics methods that permit rapid and accurate identification of molecules in a biological medium, as well microbial species from positive cultures. This method applies to biomolecules as well as peptides, proteins, glycoproteins, and oligonucleotides and it is considered more cost effective than other techniques used in microbiology. The faster identification of the pathogen and the determination of antimicrobial susceptibility is a key in the management of patients with suspected infection. Various organisms can be identified by this technique including bacteria, fungi, and parasites. Bacteria are at the top of the list of identified species succeeded by fungi and it ends with parasites. In this paper, we give a comprehensive review of MALDI-TOF MS's efficacy in identifying clinical pathogenic bacteria.

Keywords: Mass spectrometry; MALDI-TOF; Bacteria; Fungi; Parasites.

1. Introduction

Large and small biomolecules can be vaporized and ionized by matrix-assisted laser desorption ionization (MALDI) technic. As well, MALDI provides a new technology that enables the rapid and confident identification of microorganisms (Ferreira et al., 2011; Florio et al., 2018; Tanaka et al., 2017) and is now certified for regular bacterial and fungal identification in clinical diagnostic laboratories (Drissner & Freimoser, 2017). However, there is another less-used type of method called molecular typing methods that determine the genetic background of the microorganisms for their identification (Váradi et al., 2017).

The instrument's initial cost is significant; thus, it may not be in every laboratory in the world. Yet, by choosing this technic the cost savings on reagents and staffing can quickly repay the investment (Tran et al., 2015) and result in significant cost reduction (Rychert, 2019). The common approach for microbial identification in most clinical microbiology laboratories involves taking a blood sample from a positive blood culture. The main drawback of this procedure is that microbe identification occurs only after colony growth and isolation, resulting in a lengthy identification period (e.g., anaerobic bacteria and yeast) (Azrad et al., 2019). The clinical microbiology task is to help clinicians make timely and appropriate diagnostic and care decisions for infectious diseases and patients with infectious diseases. Thus, reducing the identification time for pathogen detection is very important because the time from diagnosis to appropriate antibacterial treatment has a strong

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impact on mortality (Torres et al., 2016). Bacteria, yeasts, and parasites were identified and detected in clinical samples with high specificity and high sensitivity using techniques based on MALDI time-of-flight mass spectrometry (MALDI-TOF MS) (Azrad et al., 2019).

For example, 2,032 positive blood culture samples were used to assess the accuracy of a MALDI procedure that included density centrifugation with additional chemical lysis-extraction (115 species of microorganism). With scores of 1.700, the overall MALDI-TOF MS-based identification rate was 87.60 percent. Gram-negative bacteria were reliably recognized to the genus level in 94.06 percent of cases, followed by anaerobes (93.33 percent), gram-positive bacteria (84.46 percent), and fungi (60.87 percent) (Dai et al., 2021).

Identifying pathogens and determining antibiotic susceptibility are important factors in treating patients with suspected infections. The problem in this operation is the long period of cultivation that requires 36–48 hours' laboratory time. This is a particular element that turns into essential when addressing the most pressing problem in modern healthcare, which is the rise in antibiotic-resistant germs. Furthermore, early detection of the pathogen determines the care of septic patients who require the rapid beginning of empirical treatment with broad-spectrum antibiotics, resulting in more accurate and tailored treatment. It is well established that postponement in final diagnosis and adequate antibiotic therapy can harm patient survival (Vrioni et al., 2018). Rapid microbiological diagnosis and antimicrobial susceptibility testing enable the early detection of microorganisms and resistance patterns required for the proper clinical and therapeutic management of patients (Cercenado, 2017).

MALDI-TOF-MS is widely in use in clinical microbiological diagnostics to identify pathogen species. The success of this technique has already been seen as a bacteriological revolution and has many advantages over the traditional biochemical identification of microorganisms (Welker & Moore, 2011).

The goal of this review was to describe the usefulness of MALDI-TOF MS in clinical microbiology laboratories for pathogen identification, including sample preparation and microbiological identification performance. In addition, in the clinical laboratory, recent concerns linked to MALDI-TOF MS will be presented.

2. Bacterial identification

2.1. Corynebacteria

Studies have shown that *Corynebacterium* species are significant contaminants of clinical material, but it can be difficult to make the right decision at the right time whether recovery of such bacteria means contamination or is clinically relevant (Bernard, 2012). These bacteria are gram-positive bacteria, facultative anaerobe with worldwide distribution. Pathogenic *Corynebacterium* species include *Corynebacterium diphtheriae* and nondiphtheroid (Alibi et al., 2015). *Corynebacterium* species are common skin and mucous membrane germs that are typically reported as contaminants when isolated from diverse therapeutic materials. It was, however, widely recognized as an opportunistic pathogen and identified as the cause of a variety of infectious illnesses, including pneumonia, spinal osteomyelitis, septicemia, and endocarditis (Alibi et al., 2015).

Recognition of *Corynebacterium* species is a challenge, and the difficulty increase to distinguish *Corynebacterium* species based on biochemical profiles. This is due to the constant need for many different biochemical tests nonexistent in the API Coryne system (Hahne et al., 2018; Zasada & Mosiej, 2018). Various researchers showed that MALDI-TOF-MS using the Bruker

Biotyper system is a reliable approach for the identification of *Corynebacterium* species (Alatoom et al., 2012; Vila et al., 2012). For *Corynebacterium diphtheriae*, MALDI–TOF MS had 100% concordance with the biochemical methods (Osa et al., 2021).

In the works of Alabi et al. (Alibi et al., 2015) for the identification of 97 *Corynebacterium* clinical strains recovered over 5 years from in-patient and out-patient was reported. These strains were subcultured on 5% horse blood agar incubated aerobically for 24-48h at 37℃. 1 µL of the matrix (α-cyano-4-hydroxycinnamic acid) covered an already transferred portion of a colony to a metabolomic MALDI. The first attempt of identification showed no confidence results. The matrix is then changed to formic acid 70% and then evaporated at room temperature. Measurements were performed with a Microflex mass spectrometer. The outcomes revealed that 74 out of 97 *Corynebacterium* strains of the species were identified. Out of the 23 remaining, 16 reached the confident level after the application of formic acid. A rare case of urosepsis caused by *C. riegelii*, associated originally with urinary tract infections (Funke et al., 1998), in a 67-year-old female patient with neurogenic bladder, was reported identified by MALDI-TOF (Pichon et al., 2019).

In 2021, Fong et al. reported 30 isolates of *C. macginleyi* of ocular origin from 26 patients, identified using MALDI-TOF (Fong et al., 2021). Studies approve the strength of MALDI as a powerful device, which could be applied in the clinical laboratory, developing the diagnosis of *Corynebacterium* infection (Bao et al., 2017; Gomila et al., 2012).

2.2. Staphylococci

Coagulase-positive staphylococci (CoPS) engender various diseases like bacteraemia, urinary tract infections, pyoderma, abscess, and wound infections in both humans and animals (Savini et al., 2013). Methicillin-resistant strains that have acquired *mecA* or *mecC* impart resistance to all the beta-lactams except for a few anti-staphylococci cephalosporins mainly methicillin-resistant *Staphylococcus aureus* (MRSA) which is resistant to many important drugs including fluoroquinolones and aminoglycosides limiting treatment of the infection. Various studies have shown the transmission of MRSA and other multidrug-resistant staphylococci between animals and humans (Guardabassi et al., 2004; Saputra et al., 2017).

Staphylococcus aureus contains two subspecies (*aureus* and *anaerobius*)s having distinct epidemiology and virulence. The efficiency of MALDI-TOF MS for quick identification of both subspecies was evaluated using a panel of 52 S. *aureus* isolates (30 subsp. *anaerobius* and 22 subsp. *aureus*) generated from various sources, demonstrating that it is a viable method for *S. aureus* subspecies discrimination (Pérez-Sancho et al., 2018). Nix et al. developed the application of MALDI-TOF MS based direct-on-target microdroplet growth (DOT-MGA) assay for Gram-positive bacteria including antimicrobial susceptibility testing (AST) from agar cultures and directly from positive blood cultures (BCs) using the detection of methicillin resistance as an example. The outcomes demonstrated the usefulness and efficiency of MALDI-TOF based DOT-MGA for rapid AST of *S. aureus* from agar cultures and directly from positive BCs (Nix et al., 2020).

Rapid detection of methicillin-resistant *Staphylococcus* could lead to proper medical treatment. About 400 *Staphylococcus* spp. strains were studied in order to create a reliable MALDI-TOF method for species identification.The findings show that incorporating MALDI-TOF analysis into regular laboratory work, particularly with clinical samples, could provide a quick warning in several situations regarding the existence of methicillin-resistant bacteria (Alksne et al., 2020). A recent study was conducted to assess the reliability of PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) of the *groEL* and *gap* genes to discriminate between bovine-associated NAS species, using MALDI-TOF MS as the reference method, demonstrates that *gap* PCR-RFLP is

a useful and reliable tool for the identification of NAS species isolated from bovine mastitis (Conesa et al., 2020).

2.3. Helicobacter pylori

H. pylori infects up to 50% of the world's population (Salih, 2009). These bacteria have been implicated in the causation of various gastroduodenal (duodenal ulcers, MALT lymphoma, gastric cancer) as well as extra-intestinal (e.g., refractory anaemia, idiopathic thrombocytopenic purpura) diseases (Malfertheiner et al., 2017).

In their work, Pereira et al. tend to determine the involvement of the gastric microbiota in the production of symptoms (chronic dyspepsia) in individuals with *H. pylori* infection and the nonexistence of organic disease. MALDI was used for the identification of organisms from the Bruker Biotyper 2 database. As result, in dyspeptics cases, *Staphylococcus* and *Lactobacillus* were recognized significantly frequently, while *Streptococcus*, *Pseudomonas*, *Escherichia coli,* and *Klebsiella pneumoniae* in non-dyspeptics (Pereira et al., 2018).

Gastric helicobacters (*H. pylori* and non-*H. pylori* species (NHPHs)) colonize the stomach of humans and/or animals. Since many *Helicobacter* species are recognized as bacterial carcinogens, as well as human and/ or animal pathogens, their identification is crucial (Kusters et al., 2006). Currently, only molecular techniques can distinguish *Helicobacter species*. Berlamont et al. findings's imply that MALDI-TOF MS may quickly distinguish between gastric *Helicobacter* species when a large database is available and variance owing to growth circumstances and agar-medium-related peaks are taken into account (Berlamont et al., 2021).

2.4. Mycobacteria

Mycobacteria are one of the primary types of pathogens causing a serious public health problem globally (Hou et al., 2019). Mycobacteria are widespread environmental microorganisms with over 160 species, some of which can cause lung disease, skin infections following inoculation, cervical lymphadenitis in children, and disseminated disease in severely immunocompromised people (To et al., 2020). Identification of tuberculous or even non-tuberculous mycobacteria and other acid-fast organisms such as Nocardia by MALDI-TOF posed a particular challenge. Historically, the identification has been done using time-consuming cultivation, biochemical testing, DNA probes, gas-liquid chromatography, or DNA sequencing (K. Patel et al., 2015). Before MS processing, it is necessary to inactivate living cells for safety reasons, followed by cell lysis to collect protein components for analysis(Murray, 2012). Heat inactivation at 95°C for 30 minutes can be employed to kill the cells, followed by resuspension in ethanol to a final concentration of 75 percent. Protein extraction can be done in a variety of methods. 0.5 mm zirconia/silica beads and sonication are frequently utilized, as well as extraction with 70% formic acid and acetonitrile; a combination of the two procedures is also conceivable. The complete procedure, including MALDI-TOF measurement, takes 90 minutes. (Alcaide et al., 2018). Nevertheless, MALDI-TOF identification of mycobacteria has some limits, for example, is not able to differentiate members of the *Mycobacterium tuberculosis* complex and some closely related species such as *M. chimæra* and *M. intracellulare* and others (Neuschlova et al., 2017; K. Patel et al., 2015).

2.5. Anaerobes

MALDI-TOF identification of anaerobes has become the method of choice, replacing 16S ribosomal ribonucleic acid (rRNA) gene sequencing which is considered to be the "gold standard" method for detecting sensitive and uncultivable bacteria (Coltella et al., 2013). The easiest way to identify anaerobes by MALDI-TOF is a direct smear of the sample on the MALDI target without

any extraction, but this approach often shows a low identification score which means a higher possibility of incorrect or no reliable identification. The better way is pre-extraction of proteins either on-plate by semi-extraction using 70% formic acid or off-plate extraction by 70% ethanol and 70% formic acid, to improve the quality of mass spectra (Hsu & Burnham, 2014; Stevenson et al., 2010). Bruker Microflex with Biotyper 3.0 software system was evaluated for identification of anaerobes over a collection of 101 anaerobic bacteria, counting *Clostridium* spp., *Propionibacterium* spp., *Fusobacterium* spp., *Bacteroides* spp., and auxiliary anaerobic bacterial of clinical interest. MALDI-TOF MS, for example, may accurately identify anaerobes produced in less-than-ideal conditions, such as on selective culture media or after exposure to oxygen (Hsu & Burnham, 2014).

A meta-analysis carried out by Li et al. covering 6685 strains of anaerobic bacteria, conclude that MALDI-TOF MS was accurate at 84% for species ($I2 = 98.0\%$, $P < 0.1$), and 92% for genus ($I2 = 96.6\%$, $P < 0.1$) (Li et al., 2019). However, the two MALDI devices performed differently. The VITEK MS system had a 90 % identification accuracy rate compared to 86 % for the MALDI biotyper system (Li et al., 2019).

During the work of Alcalá et al., MALDI-TOF MS is effective in identifying anaerobes, with a strong correlation to phenotypic and traditional approaches. Only 2.1 % of the isolates could not be correctly identified in the time of the work, imposing the use of genetic techniques for definitive recognition (Alcalá et al., 2021). However, this method still has some drawbacks in accurately identifying the types of rare and common anaerobes (Li et al., 2019).

2.6. Gram-negative bacteria

MS v2.0 MALDI system was evaluated in the identification of gram-negative bacteria in a multicenter study. The final results have shown that VITEK MS system produced a solid, species-level identification for 96 % of 226 isolates when compared with the reference method (DNA sequencing); an additional 1% were accurately identified to the genus level (Branda et al., 2014). Moreover, traditional phenotypic methods (Phoenix™) directly from 318 BACTEC™ blood cultures were compared with MALDI identification of Gram-negative bacteria. The MS score was ≥1.7 in 268 (91.8%) monomicrobial broths, with concordance to genus and species levels of 100% and 97.0%, respectively. A limitation in the capacity of MALDI-TOF MS to detect all species in polymicrobial broths remains present (Gray et al., 2013). Faron et al. concluded that MALDI-TOF MS technic succeed in distinguishing 99.8% (2258/2263) to genus and 98.2% (2222/2263) to species level of all obtained from different sources (Faron et al., 2015).

Additionally, MALDI cannot identify some pathogens, such as *Streptococcus Viridans* and encapsulated microorganisms like *Klebsiella pneumoniae* and *Haemophilus influenza*. When BCbroth is evaluated directly, the components of human blood can induce interference or provide noisy spectra. As a result, pre-processing the BC broth is critical for reducing interference and concentrating the bacteria. The MBT SepsityperTM kit was created to address these issues. More Gram-negative organisms (100 % for genus and 91.4 percent for species level) were accurately identified in one investigation using the SepsityperTM kit than Gram-positive organisms (100 for genus and 67.7 percent for species level). Anaerobic bacteria and alpha-hemolytic streptococci were shown to be difficult to treat (Hou et al., 2019).

3. Detection of resistance mechanisms: β-lactamase

MALDI-TOF MS is also used for antimicrobial susceptibility testing. The growing number of antibiotic-resistant microorganisms is an increasing health care problem (Florio et al., 2018). MALDI-TOF MS, is able to detect the β-lactamase. Using this instrument, β-lactamase resistance is not directly determined, however, the chemical reaction of hydrolysis of the β-lactam ring by β-lactamase is determined. If the β-lactam antibiotic is hydrolyzed by β-lactamase, then the molecular weight of the antibiotic will change (+18 Da). After this hydrolysis, carboxylation will happen (-44 Da). The methodology would entail culturing a target bacterial isolate in the presence of a β-lactam antibiotic for 1 to 3 hours, then testing the culture upper layer for the antimicrobial hydrolysis product. Because of the many manipulations required and the variety of antimicrobial targets, this approach may not be able to substitute all antimicrobial susceptibility tests (cases where no direct metabolism of the antibacterial cannot be detected using this technic) (Ledeboer & Hodinka, 2011).

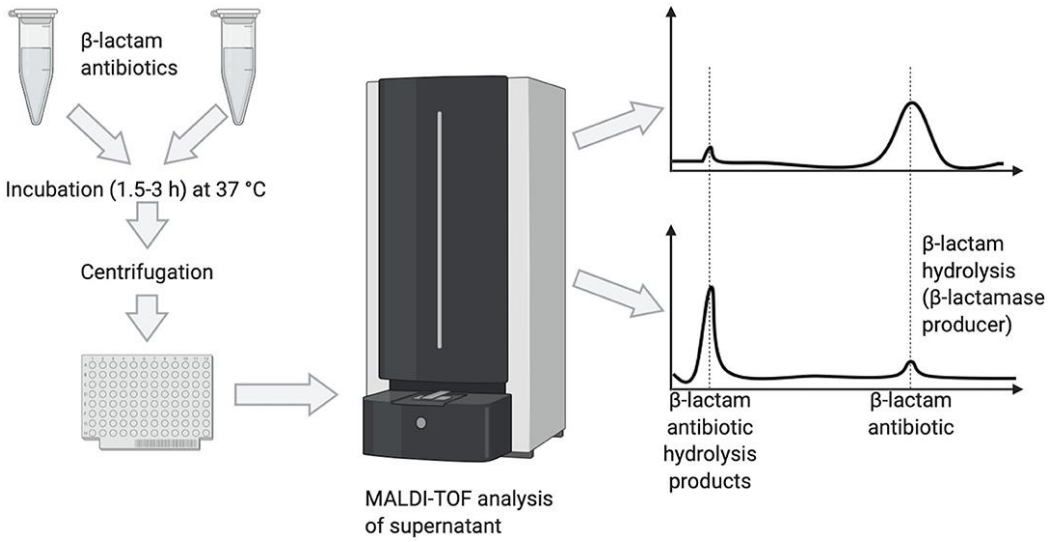

Figure 1. Detection of β-lactamase producers by MALDI-TOF MS based on the hydrolysis of the target β-lactam antibiotic, as visualized by peak disappearance (Florio et al., 2018).

4. Fungal identification

Similar bacteria, fungi may be tested in two ways. The first is "direct transfer," in which whole cells from colonies are transferred to a "spot" on a MALDI-TOF MS target plate, with or without on-plate treatment using formic acid in order to lyse cells, also known as "on-plate extraction" and "extended direct transfer". In the case of the second, a more formal (and time-consuming) off-plate protein extraction process was used. Yeasts are generally labeled as the former, while filamentous fungi are labeled as the latter. Yeasts are generally labeled as the former, while filamentous fungi are labeled as the latter. Knowing that yeasts and filamentous fungi have minor mass peaks and minor reference spectra than bacteria, MALDI biotyping of yeasts and filamentous fungi is more complicated. In contrast to clinically important fungi, MALDI biotyping has only found a small number of fungal plant diseases or food pollutants. (Drissner & Freimoser, 2017; R. Patel, 2019).

4.1. Yeast

Recent research evaluating MALDI-TOF MS to DNA-based gold-standard approaches for the identification of over 1,000 yeast isolates (>95 percent Candida) suggests that effective species identification ranges from 95 to 98 percent (Cassagne et al., 2016; Wang et al., 2016). MALDI-TOF MS was described with yeast in 2001 (Amiri-Eliasi & Fenselau, 2001), and rapidly become a standard method for yeast identification especially *Candida* and *Cryptococcus* species (R. Patel, 2019). Even closely related yeast species which cannot be discriminated with common biochemical methods such as those of the *Candida ortho-/ meta-/ parapsilosis* complex can be resolved without difficulty (Bader et al., 2010). Meena et al. released a study that compared MALDI-TOF-MS to traditional methods for identifying therapeutically relevant yeasts (germ tube or morphological appearance on cornmeal agar (CMA), biochemical procedures employing API kits, and molecular biology techniques) (Meena et al., 2021). The overall results demonstrate that, while there was a significant association between the two approaches, MALDI-TOF-MS successfully estimated the most commonly isolated bloodstream Candida species, including *Candida albicans*, *C. tropicalis*, *C. parapsilosis*, *C. lusitaniae*, and *C. glabrata*. Ceballos-Garzón et al. compared the performance of MALDI-TOF MS versus MicroScan in the identification of emerging and multidrug resistant yeasts in a fourth-level hospital in Bogotá, Colombia (Ceballos-Garzón et al., 2019). In the case of 498 yeast isolates, the percentage of agreement between the two approaches was 93.6 percent (32 discrepant isolates). The ITS sequencing consistency with MALDI-TOF MS was stronger (99%) than the MicroScan concordance (94 percent). Consequently, the need for MS and molecular approaches such as MALDI-TOF MS and ITS rDNA barcoding for accurately identifying new or cryptic yeast species was emphasized; also, some of them may be multidrug-resistant.

Despite prior research that used off-plate yeast extraction, on-plate formic acid extraction is now preferred due to its ease of use; on-plate formic acid preparation produces greater recognition percentages over direct transfer solely (figure 6) (R. Patel, 2019). MALDI-TOF MS-based identification of yeast requires pretreatment of the sample before the acquisition of the spectra. The most commonly used is on-plate protein extraction by 70% formic acid or complex procedures can be used more such as ethanol/formic acid extraction or formic acid/acetonitrile extraction (Cassagne et al., 2013; Quintilla et al., 2018).

4.1.1. Malassezia

Malassezia species are opportunistic pathogens, causing various skin diseases, such as pityriasis versicolor, seborrheic dermatitis, or dandruff strains isolated from skin, urinary, fecal, respiratory, and blood samples and identified as *Malassezia* (Sugita et al., 2001). Denis et al. (Denis et al., 2017) conducted a study in which 45 strains were grown for 10 days on Dixon medium at 32°C. After dyeing with methylene blue, the microscopic morphological study enables genus determination. By sequencing the internal transcribed spacer, all strains have been reported to the species level. Microflex mass spectrometer showed that no spectra could be acquired when the strains were cultivated on Dixon medium. As a result, each strain was subcultured on Sabouraud chloramphenicol agar with 0.5 percent commercial organic olive oil. Mass spectra were repeatable using 2- to 5-day-old colonies and a protein extract kept at 4°C under similar circumstances. A total of 85 Malassezia isolates were studied from patients in three French university hospitals. Internal transcribed spacer sequencing was used to identify each strain. A MALDI-TOF database was created using 45 strains from the six species *Malassezia furfur*, *M. sympodialis*, *M. slooffiae*, *M. globosa*, *M. restricta*, and *M. pachydermatis*. This information was tested with forty different strains. According to the manufacturer's requirements, all strains with log scores of >2.0 were identified by our Malassezia database. The log score values have a coefficient of variation of less

than 10%, according to repeatability and reproducibility tests. Finally, this new *Malassezia* database makes it simple, quick, and accurate to identify *Malassezia* species. The use of this database will aid in the better and quicker identification of *Malassezia* species, as well as a better knowledge of their epidemiology (Denis et al., 2017).

Table 1. Means of the coefficients of variation of the log score values of the reproducibility and repeatability tests (Denis et al., 2017).

	Mean (range) CV (%) of log score values		
Age of colony (days)	Analysis repeatability	Full-protocol repeatability	Reproducibility
2	4.2 (3–6)	4.5 (3–6)	5.4 (2–7)
3	3.6 (3–5)	6 (5–7)	4.8 (4–8)
4	4.2 (3–6)	4.5 (4–5)	5.8 (3–9)
5	4 (3–6)	4.5 (4–5)	6.6 (5–8)

The work of Honnavar et al. aimed to improve the identification of *Malassezia* species using MALDI (Honnavar et al., 2018). For database development and validation, a total of 88 isolates were employed, with 190 isolates confirmed by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCRRFLP). The new *Malassezia* database correctly identified 94.7 % and 5.3 % of strains to the species and genus level, respectively, and the results were comparable to PCR-RFLP with a kappa value of 0.9 (Honnavar et al., 2018).

4.1.2. Trichosporon

Trichosporon, opportunistic non-*Candida* yeasts, have recently emerged as significant human pathogens (Chitasombat et al., 2012; de Almeida Júnior & Hennequin, 2016) and the identification of these species is important, both for epidemiological purposes and for therapeutic management.

Ninety-three Trichosporon strains were included in a study (de Almeida Júnior et al., 2014) and the mass spectra were acquired using a Microflex LT MALDI–TOF mass spectrometer. The first comparison of three different protein extraction protocols was done to determine which led to spectra with the highest log scores. The isolates were subcultured for 48 hours at 30°C on Sabouraud dextrose agar with chloramphenicol and gentamicin. For the colonies taken from the slant cultures, all three of the studied procedures depended on the extraction efficacy of ethanol 70% and formic acid 70% plus acetonitrile 100%. For yeast identification, the first approach followed the manufacturer's instructions. The time of initial centrifugation, the length of incubation, the volumes of formic acid and acetonitrile utilized, and the volume of the final deposit on the slide were all different.

Using a broad variety of strains and isolates from different yeast collections in Brazil and France, De Almeida et al. analyzed and enhanced Vitek MS for the identification of common clinically significant species of *Trichosporon*, *Cutaneotrichosporon*, and *Apiotrichum*. Light of the findings, the former SuperSpectra from the SARAMIS version 4.13 database (bioMérieux) that were linked to misidentifications were deactivated, and the improved database with the in-house SuperSpectra was put to the test for strain and isolate verification (de Almeida et al., 2017).

4.2. Filamentous fungi

Infections produced by filamentous fungus (moulds) are aggressive in people with weakened immune sytems and pose a significant concern to the increasing number of immunocompromised patients, particularly those with neutropenia. Diabetes, hematologic

malignancies, iron overload, human immunodeficiency virus (HIV) infection, solid organ grafts, and significant burns are all at risk. Because fungal infections are associated with high mortality and morbidity, rapid and accurate identification of clinical isolates is crucial (Becker et al., 2014). The increased interest has risen in applying MALDI method to filamentous fungi (Normand et al., 2013; Ranque et al., 2014).

Moulds possess phylogenetic relationships closer than among yeasts (Samson & Varga, 2009) and here species-boundaries are not drawn as easily. In other words, filamentous fungi have a wide range of phenotypes, which can lead to a wide range of protein spectra; diversity can be influenced by growth conditions and the section of fungal mycelium studied. Nonetheless, MALDI-TOF MS can be used to identify the filamentous fungus.

In the case of the identification of filamentous fungi, the gold standard is based on morphologic features and DNA sequencing. However, DNA sequencing is expensive, labor-intensive, and is vulnerable to contamination due to its multistep workflow (Becker et al., 2014). Identification of filamentous fungi by MALDI-TOF is complicated because fungal cultures are heterogenic. Whereas spectra made from different morphologies may vary significantly (Alanio et al., 2011). They don't only consist of different cell types such as mycelium, spores, and fructification organs, but there is also a significant difference between the center of a colony and the periphery, and it also depends on the age of the culture. The solution of this should be the use of protein isolation from the culture suspension rather than from their colonies grown on plates. Also, on-plate extraction with organic acids such as formic acid can be used (Hendrickx, 2017; Sanguinetti & Posteraro, 2017).

5. Parasites identification

In recent years, established techniques for identifying microbial species, MALDI-TOF MS profiling or proteomes based on MALDI typing, have also been successfully applied to a wide variety of parasites and their vectors (Murugaiyan & Roesler, 2017). Despite that, some works have also reported the use of MALDI-TOF MS for the identification of parasites, like *Leishmania*, *Giardia*, *Cryptosporidium*, *Entamoeba*, ticks, and fleas (Singhal et al., 2016). *Cryptosporidium* spp. linked with human infections was the subject of the primary report of MALDI typing of protozoa. Oocytes of *C. parvum* and *C. muris* were extracted from feces of experimentally inoculated mice, lysed by freeze-thaw cycle, and spotted using HCCA as the matrix to provide a specific spectrum for each species (Magnuson et al., 2000). Later, intact oocytes and purified sporozoite can be incubated with the matrix for 45 minutes. It is important in generating a mass spectrum containing many reproducible peaks of *C. parvum* oocysts. Then apply the whole spores, spore coat, and soluble fraction of spore-forming single-cell parasites such as microsporidia, *Encephalitozoon cuniculi*, *Encephalitozoon bright*, *Encephalitozoon intestinalis*, *Brachiola algerae* directly. They are isolated from humans and propagated on monolayers of Vero monkey kidney (E6) cells, displayed species-specific markers in the mass range (E6) of 2,000 to 8,000 Da. Subsequently, specific peaks related to species in the region of *m/z* 3,000–19,000 were discovered for *Giardia* spp., the water-borne protozoan parasite that causes giardiasis. *G. lamblia* and *G. muris cysts*, cyst walls, and trophozoites were extracted from the feces of experimentally challenged mice, washed, combined with an equal volume of matrix solution (sinnapinic acid), incubated, and spotted onto a MALDI plate for analysis (Murugaiyan & Roesler, 2017).

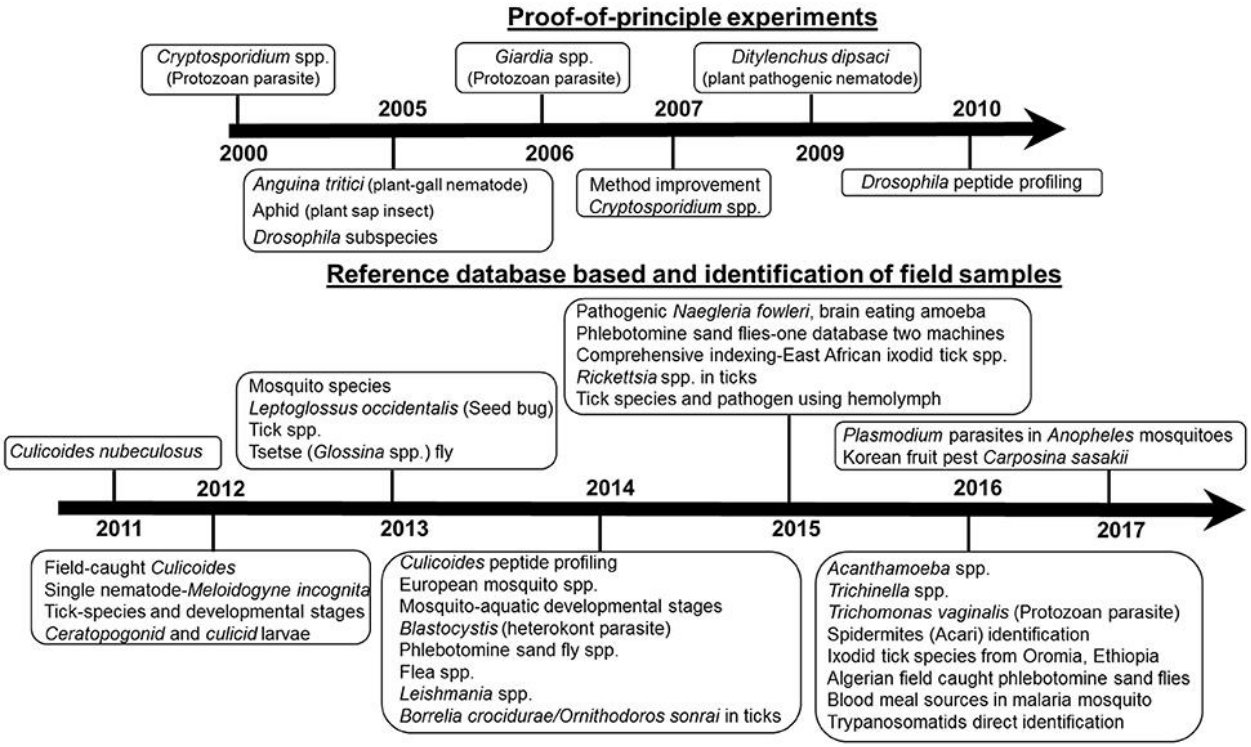

Figure 2. Chronology of MALDI profiling application of pests, parasites, and vector species identification (Murugaiyan & Roesler, 2017).

Currently, MALDI can successfully identify the majority of pathogens. Despite that, MALDI has less exact for the distinguish of pathogens having similar mass spectra (Chen et al., 2021). Out of these pathogens are several strains of a complex group of bacteria, including *A. calcoaceticus-A. baumannii* complex, *Enterobacter cloacae* complex, *Burkholderia cepacia* complex, and *S. mitis* complex (Cuénod et al., 2021; Sousa et al., 2014; van Prehn et al., 2016; Wong et al., 2020).

MALDI was applied to distinguish lice from animal agriculture in Algeria (Ouarti et al., 2020). Fourteen species were tested, among them we cite *Bovicola bovis*, *B. ovis*, *B. caprae*, *Chelopistes meleagridis*, *Goniocotes gallinae*, *Haematopinus eurysternus*, *Linognathus africanus*, *L. vituli*, *Solenopotes capillatus*, *Menacanthus stramineus*, *Lipeurus caponis* and *Pediculus humanus corporis*. Results suggested MALDI as a potent device for differentiating lice species with scores ranging from 76 % to 100 % (Ouarti et al., 2020).

Overall, we list some parasites which have been identified using MALDI-TOF MS: *Leishmania* spp. (Cassagne et al., 2014; CULHA et al., 2014; Mouri et al., 2014), *Cryptosporidium* spp. (Fei et al., 2018; Gathercole et al., 2021), *Giardia* spp. (Villegas et al., 2006), *Blastocystis* spp. (de Dios Caballero & Martin, 2018; Martiny et al., 2014), *Trichomonas vaginalis* (Calderaro et al., 2016), *Entamoeba histolytica* (Ng et al., 2018; Ujang et al., 2019), Ticks spp. (Boucheikhchoukh et al., 2018; Diarra et al., 2017; Huynh et al., 2021; Yssouf et al., 2015) and Flea spp. (Hamzaoui et al., 2018, 2020; Yssouf et al., 2014).

Finally, despite the higher expenses of MALDI-TOF introduction total hospital expenditures, for instance, dropped by $2,439 per bloodstream infection, resulting in a $2.34 million yearly cost savings (T. S. Patel et al., 2017).

6. Conclusion

Despite the high cost of this technique, using molecular methods in the routine workflow of clinical laboratories has significantly decreased the time required for microbiological outcomes and significant cost saving can be achieved. Microbial identification based on MALDI-TOF MS has evolved into a method that can be easily applied to routine clinical diagnosis. MS-based identification is the presence of MALDI-TOF MS equipment in laboratories around the world, as it has the potential to extend to other areas of microbiology, ultimately entomology, ecology, parasitology, and other disciplines. We have reviewed in this paper the variety of applications of MALDI for microorganism identification. Overall, MALDI TOF MS has been demonstrated to be an easy to perform, an accurate, reliable, and rapid method in microbiology laboratories.

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Conflict of interest

The author declared no conflict of interest.

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