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RESEARCH ARTICLE

APPLICATIONS OF MALDI TOF-MS IN CLINICAL MICROBIOLOGY LABORATORY

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ABSTRACT

Matrix Assisted Laser Desorption Ionization of Time of Flight Mass Spectrometry (MALDI-TOF MS) is an emerging technology for Clinical Microbiology Laboratory. In most of the Microbiology Laboratories, The identification of microorganisms is done by phenotypic method conventionally or by molecular methods in most of the Microbiology Laboratories even today. The phenotypic methods are based on isolation of the microorganism in culture followed by biochemical tests for bacteria which are time consuming. The molecular methods require expertise and new variants may not be detected. Moreover, rapid and accurate identification of bacteria is necessary for diagnosis and efficient treatment of the patient which may be life saving. MALDI-TOF MS is an easy, rapid and efficient method for identification of bacteria which are even difficult to grow, typing of bacteria, fungi and viruses. In this review, the principle, mechanism, uses, limitations and future perspectives of MALDI-TOF MS have been discussed.

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INTRODUCTION

In Laboratory Medicine, Twenty first Century is the era of evolution of automated methods. Even most of the Biochemistry and Pathology Laboratories in India are using automated and semi automated methods but Microbiology laboratories are yet to adopt the automated methods in day to day practice. As the machine cannot think or exercise and moreover are expensive, very little option is left for Laboratory personnel of Clinical Microbiology. Every where the man behind the machine is more important not only for interpretation of reports but for proper working of machine, Automated methods are slowly emerging for Clinical Microbiology Laboratories for detection of growth, rapid identification of fastidious organisms, antimicrobial susceptibility testing and genotyping etc. Matrix- assisted Laser desorption / ionization time-of-flight mass spectrometry (MALDI TOF MS) is a newer technology for identification of bacteria, fungi and viruses in Clinical Microbiology laboratory(Bizzini and Gerub, 201`0).It has opened a new era of Automation in Clinical Microbiology and brought us from conventional biochemical identification to MALDI TOF MS which is based on protein biomarkers, mainly 16s ribosomal proteins (Tanaka *et al.*., 1988). In 1975, Anhalt and Fenselau described first time the use of Mass spectrometry for bacterial characterization (Anhalt and Fenselau, 1975). Matrix assisted laser desorption ionization process was introduced in late 1980s. In 1988, Michael Karas and Franz Hillenkamp coined the name MALDI (Karas and Hillenkamp, 1988). The development of time-of-flight vacuum tube made detection of proteins of mass more than 100 kDa possible and it was reported by Hillenkamp at the Boredeaux International Mass spectrometry meeting (Hillenkamp,1989). In 1989, the first practical MALDI-TOF MS was built by Beavis and Chait (Beavis and Chait, 1989) and then it has undergone gradual evolution. MALDI-TOF MS was used in Clinical Microbiology Laboratory in Europe in mid 1990s. From 1994 to 1996 several groups used MALDI-TOF for protein profile after cellular extraction and purification (Cain *et al.*.,1994; Girault *et al.*,1996; Liang *et al.*, 1996). The first complete database for bacterial identification by MALDI-TOF MS was reported in 2004 (Keys *et al.*,2004). This new technology has been proven as potential tool for microbial identification including antimicrobial resistance in microorganism which helps to start effective therapy to patients.

Principle and mechanism: MALDI is based on soft ionization technology which allows ionization and vaporization of large non-volatile protein molecules (Emonet

et al., 2010). This generates single charged ions so that mass-to-charge ratio (m/z) of the bioanalyte corresponds to its mass value. MALDI-TOF MS has three basic principal units. In the first unit, the analyte is embedded with crystal matrix, where laser beam causes ionization and transfers ions into gas phase. The second unit contains mass analyser which allows ion separation as per mass-to-charge ratio (m/z). The third unit has detection device which monitors separated ions. Several methods are available for sample preparation for MALDI-TOF MS which include putting bacterial growth onto the target plate with or without addition of an acidic solution and other method include extraction of protein from bacteria. The second method is cumbersome and complicated but still reserved for processing of difficult to lyze and fastidious organisms. The first method i.e. direct colony testing is easy, faster, user-friendly and cost effective. A colony grown on culture plate is taken and is put into the well present on metallic target plate which contains multiple wells. The formic acid is added to the well to enhance the generated mass spectrum. The mixture is allowed to dry and then target plated is placed in Mass Spectrometry ionization unit. In MALDI, sample is mixed with crystalloid matrix solution (e.g. α- cyanoixed-4-hydroxycinnamic acid dissolved in 50% acetonitrile mixed with 2.5% trifluroacetic acid). It is the very important first step using MALDI-TOF-MS. The main role of matrix (CHCA for Bruker instrument and DHB for Vitek MS system) are it helps to break the cell wall, crystallizes proteins within seconds and protects protein from fragmentation by LASER. When the matrix is exposed to LASER beam, the matrix absorbs the light energy and transfer this energy to protein molecules for ionization. These ions are then accelerated in an electrostatic field into high vacuum flight tube, until they reach the detector. Smaller ions travel faster than larger ones. The time of flight requires to reach detector is dependent on mass and charge of bio-analyte (Jasna, 2007), resulting in a spectral profile compared with reference databases which is specific for a given species. There are large numbers of ribosomal proteins, which contribute to generate mass spectra, and those are unique to individual organism type. The peaks produced are specific to genus, species and strain. These peaks of the generated mass spectrum of test isolate is compared to reference database spectra and identification is based on the most closely relatedness.

According to Sigma Aldrich, the matrix must have the following properties such as it should be able to embed and isolate analytes by co-crystallization, soluble in solvents compatible with analyte, vacuum stable, absorb the LASER wavelength, cause co-desorption of analyte after LASER irradiation, promote analyte ionization etc (http://www.sigmaaldrich.com/img/assets/4242/fl_analytix6_2001). LASERs used in MALDI: Numerous gas and solid state LASERs have been developed for MALDI. Most MALDI devices use a pulsed ultra-violet (UV) LASER-N2 source at 337 nm and Neodymium-yttrium aluminium garnet (Nd: YAG) which emits at 335 nm and gives a longer pulse time. Infrared (IR) LASERs are also used for MALDI. The most commonly used IR LASER is the ebium doped-yttium aluminum garnet (ER: YAG) which emits at 2.94μm, softer than UV and useful for certain biomolecules but matrices available for IR absorption are limited. The existing previous tools like PROTEO, NEAPOLIS and Geena can be used for analysis of MALDI-TOF MS spectra but recently Geena 2 has been developed which can be used as a public tool for automated preprocessing of MS data originated by MALDI-TOF) (Romano *et al.*, 2016.

Uses of MALDI-TOF in clinical microbiology: MALDI-TOF MS can be routinely used for microbial identification and strain typing of bacteria, fungi and viruses. It can be also used for epidemiological studies, detection of Biological warfare agents, detection of water & food-borne pathogens, detection of drug resistance, mutation of viruses etc. Recently, MALDI-TOF is used for detection of pathogens from direct samples.

Bacterial identification: Conventional laboratory techniques for identification of different organisms are based on microscopy, cultural characteristics, biochemical tests and detection of antigen etc. Recently molecular methods are being used for identification of organisms. These all are time-consuming and require expertise. MALDI-TOF MS allows quick characterization of wide variety of microorganisms such as bacteria, fungi and viruses, within minutes to few hours and it is also a potential alternative to conventional methods and molecular methods. In routine identification of bacterial colonies on culture plates, it gives 100% identification for *Neisseria, Mycobacteria, Salmonella, Helicobacter pylori & Campylobacter species, Staphylococcus aureus* and some species of Coagulase Negative Staphylococus (CONS) (Wieser,*et al.*, 2012). MALDI-TOF MS gives 97.7% of identification rate for Enterbactericeae, , 84% for HACEK group and >90% for anaerobic bacteria (e.g. *Bacteroides sp., Clostridia sp, Actinomyces sp., Prevotella sp., Fusobacteriumr sp.*). Bacterial strain typing can also be done using MALDI-TOF MS. It can detect Slow growing or Fastidious bacteria like *Bartonella sp., Legionella sp., Coxiella burnetti, Mycobacteria sp., Arachea sp.* (environmental pathogen), Food & water borne pathogens like Aeromonas sp. (Jamal *et al..*,2013). Rapid and accurate identification of Aeromonas sp., which causes severe infection through contamination of drinking water, can also be done by MALDI-TOF MS. MALD-TOF MS can identify *Bacteroides fragilis* very specifically e.g. using VITEK MS system the identification rate is 100% (Bizzini *et al.*,2010). Other anaerobic bacteria like *Clostridia sp., Prevotella, Fusobacteria, Treponema, Pepstreptococcus and Porphyromonas* etc. can also be identified by MALDI-TOF MS.

Detection of antimicrobial resistance: MALDI-TOF MS can be used to detect *Methicillin Resistant Staphylococcus aureus (MRSA)* and its subtypes (Croxatto *et al.*, 2012). Similarly MALDI-TOF MS can be used to detect *Vancomycin Resistant Enterococci (VRE)* and specially to screen *Vancomycin Resistant Enterococcus faecium* from *Vancomycin Sensitive Enterococcus faecium* (Nakano *et al.*, 2014; Wang *et al.*, 2014). The commonest mode of microbial resistance to β-lactam drugs is due to production of β-lactamase enzyme which can be detected by MALDI-TOF MS using a 'mass spectrometric β-lactamase (MSBL) assay'. Using MSBL assay β-lactamase producing strains of *E.coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Acinetobacter baumanii, Citrobacter freundii* have been detected (Hooff *et al.*, 2012; Kostrzewa *et al.*, 2013). It has been reported that detection of carbapenemase production in anaerobic bacteria like *Bacteroides fragilis* could be done in 2.5 hours (Johnson *et al.*, 2014). Carbapenem resistant Enterobacteriaceae (CRE) and Metallo β-lactamase (MBL) producing *Pseudomonas aeruginosa* could be identified by using MALDI-TOF MS (Hoyos-Mallecot *et al.*, 2014). A modified method consisting

of extraction of periplasmic space in solution and digested with trypsin had been successfully used to detect CTX-M-1 an extended spectrum β-lactamase β-lactamase (ESBL), VIM a MBL and CMY-2 an AmpC β-lactamase (Hart *et al.*, 2015). Similarly aminoglycoside resistance can be detected in future.

Disadvantages in identification of bacteria: Shigella is not differentiated by MALDI biotyper as it is considered as phylogenetical part of E.coli and gives no different pattern (Seng *et al.*, 2009). Genus Acinetobacter is repotrted as Acinetobacter baumanii- calcoaceticus complex and no species differentiation could be done. There is also misidentificaton of Streptococcus pneumoniae and Streptococcus mitis/ Streptococcus oralis (Van Veen *et al.*, 2010; Saleeb *et al.*, 2011) Bordetella pertusis and Bordetella bronchoseptica, Stenotrophomonas matophila & Pseudomonas sp. (e.g. P, hibiscola, P geniculata, P. beteli) as they have more similarities in their ribosomal protein sequences and taxonomical discordance. Propionobacterium acne are wrongly identified as Eubacterium brachy.

Detection of Mycobacteria: Identification of Mycobacteria and other acid-fast organisms is very challenging by MALDI-TOF MS due to safety reasons and inadequate methods for cell lysis. This problem can be resolved by developing special techniques such as heating bacterial suspension at 95^0 C for 30 minutes, using micropestle for dispersion of bacteria, vortexing the suspension with glass beads for lysis in presence of formic acid and acetonitrile (Lotz *et al.*, 2010). Identification of Mycobacteria takes around 90 minutes, which is quite faster compared to gene sequencing or biochemical tests which requires days to weeks. A mycobacterial database could be prepared by using selected Mycobacterial strains and comprising species specific spectral profiles (Hettick *et al.*, 2004). Under the preanalytical and analytical conditions used in one study, *Mycobacterium abscessus, Mycobacterium massiliense and Mycobacterium boletti and Mycobacterium tuberculosis complex (M.tuberculosis, M.bovis, M. microti and M. africanum)* produced similar mass profiles due to their high degree of genetic similarity. But many studies have demonstrated MALDI-TOF MS as good alternative with respect to high reproducibility and specificity to other time consuming and fastidious conventional mycobacterial identification methods (Lefmann *et al.*, 2004; Lotz *et al.*, 2010; De Carolis *et al.*., 2014). Similar to *Mycobacteria, Nocardia and Actinomycetes* have complex cell wall and need special treatment when analysed in MALDI-TOF MS.

Identification of fungus: In MALDI-TOF MS the fungal identification is mainly based on 18s ribosomal protein sequences. The sample preparation is important and extra efforts are needed as fungi possess thicker cell wall compared to bacteria. Fungal growth is inoculated in 70% ethyl alcohol and the suspension is pelleted, allowed to dry and resuspended in 70% formic acid and acetonitrile. Then the suspension is centrifuged and 1µl supernatant is smeared on Bruker MALDI system's target plate and matrix is covered for further analysis. But in Vitek MS system, direct fungal colonies are smeared on target plate, then 25% formic acid is used for lysis and matrix is applied (Pinto *et al.*, 2011). Several species of Candida such as *C. albicans, C. guillermondii , C. kefyr, C. orthopsilosis and C. parapsilosis and Cryptococcus neoformans & Cryptococcus gattii* can be accurately identified (Posteraro *et al.*, 2012). But thicker cell wall, variation in phenotypic structural changes, secondary metabolite production e.g. aflatoxin and agar contamination of analyte etc. make identification of filamentous fungi more difficult (Hibben *et al.*, 2007; Alanio *et al.*, 2011). However some improvements have been observed in identification for *Aspergillus, Penicillium, Fusarium* and dermatophytes (Santos *et al.*, 2010). It is difficult to detect the antifungal drug resistance and fungal strain typing by MALDI-TOF MS. Fluconazole resistance in Candida albicans have been successfully done in few studies (Marinach *et al.*, 2009; Saracli *et al.*, 2015).

Identification of viruses: Commonly viral infection are detected in Laboratory, by serological tests eg Enzyme linked Immunosorbent assay (ELISA) or Immunofluorescent assay or Immunohistochemistry etc. and in recent years by Molecular methods such as Polymerase Chain Reaction (PCR) or Dot Blot hybridization. Though tissue culture is gold standard for laboratory detection of viruses it is very cumbersome, take long time and even some viruses cannot be cultured e.g. Hepatitis B virus (HBV), Hepatitis C virus (HCV) etc. Hence, there is always a search for laboratory technique which should be easy and less time consuming. MALDI-TOF MS has been introduced in clinical virology recently. The inherent problem of MALDI-TOF MS for detection of viruses are mainly low protein content of viruses (Kilem *et al.*, 2012), viral proteins are usually of high molecular weight and carryover of cells when viruses are grown in cell culture. The applications of MALDI-TOF MS in Clinical Virology are – identification of viruses in clinical specimens, identification of antiviral drug resistance, detection of mutant variants, genotyping of viruses and for surveillance of viral infections. There are some reports that MALDI-TOF MS was used for detection of viruses like hepatitis virus, influenza viruses, herpes viruses, human papilloma viruses (HPV) etc. (Yi *et al.*, 2011; Piao *et al.*). The viral genetic material was amplified by PCR and the amplicons were identified by MALDI-TOF MS. Piao *et al.* has reported that by using PCR mass assay (combination of Multiplex PCR with MALDI-TOF MS) eight human enteric viruses i.e. Hepatitis E virus, Coxsackie virus, Polio virus, ECHO virus, Noro virus, Astrovirus and Reovirus could be detected simultaneously (Duu *et al.* 2011). High risk Human Papilloma viruses can be detected by using mass array technique based on MALDI-TOF MS (Hong *et al.*, 2004). Rapid and accurate epidemiological data can be provided by MALDI-TOF MS for infection control in Health care set up in case of outbreak.

Viral genotying: MALDI-TOF MS can detect the YMDD mutants and 60 Hepatitis B Virus (HBV) variants (Luan *et al.*, 2009; Oh *et al.*, 2008). Genotyping of Hepatitis C virus (HCV) and JC virus can also be done by MALDI-TOF MS (Baylis *et al.*, 2010; Yea *et al.*, 2011). It can be used for detection of mutations (H5) in Influenza A virus (Zircher *et al.*, 2012).

Detection of virral drug resistance: PCR based MALDI-TOF analysis has been used to detect Ganciclovir resistance in Cytomegal viruses that can infect transplant recepient (Cobo , 2013). It has also been reported to detect Lamivudine resistance in HBV using MALDI-TOF MS and this s method can be used for detection of HBV mutants and monitoring of antiviral therapy in chronic HBV cases (Papadopoulos *et al.*, 2004).

Identification of biomarkers in parasitic diseases: Many workers have used surface-enhanced laser desorption

ionization time of flight mass spectrometry (SELDI-TOF MS) for identification of parasitic diseases like African trypanosomiasis (Rioux *et al.*,, 2008), fascioliasis (Deckers *et al.* ,2008), cysticercosis (Ndao,2009) and Chagas diseases. In these studies, serum proteins were detected which are specific for a particular disease and described as proteomic fingerprint (Lasch *et al.*, 2009). The SELDI, a derivation of MALDI, allows sample binding to chemically active Protein Chip surfaces. SELDI has lower resolution and is unsuitable for high molecular weight proteins (>100 kDa) compared to MALDI (Lasch *et al.*, 2009).

Identification of Biological Warfare Agents: In biological warfare, early detection of agent is must to start treatment measures. MALDI-TOF MS can identify *Bacillus anthracis, Coxiella burnetti, Francisella tularensis, Yersinia pestis* etc.within minutes to hours (Shaw *et al.*, 2004; Pierce *et al.*, 2007; Ayyadurai *et al.*,2010; Seibold *et al.*, 2010; Lista *et al.*, 2011; Vranakis *et al.*, 2013; Kull *et al.*, 2010). The toxins which are used for biological warfare like Staphylococcal enterotoxin, Botulinum neurotoxin, *Clostridium perfringens* toxin, Shiga toxin can also be detected by MALDI-TOF MS (Alam *et al.*, 2012; Lasch *et al.*, 2008). Several protocols have been developed for inactivation of vegetative spore and highly infectious microorganisms. In 2008, Lasch *et al.* reported the use of Trifluroacetic acid (TFA) for inactivation of spore Couderc *et al.* in 2012, reported that for Yersinia, ethanol was more effective than TFA (Couderc *et al.*, 2012). In 2014, Jeong *et al.*. reported that detection and identification of aerosolized Bacillus spores without any pretreatment are smeared on target plate and was dried then matrix were applied and lastly were analysed by MALDI-TOF MS (Jeong *et al.*, 2014). In food Microbiology, MALDI-TOF MS has importantt role in identifying lactic acid bacteria in fermented food products, in milk products and pork (Nguyen *et al.*,2013; Nicolaou and Goodacre , 2012) Identification of pathogens contaminating infant feed e.g. Cronobacter (Stephan *et al.*, 2010) and sea food can also be done by MALDI-TOF MS (Hazen *et al.*, 2009; Bohme *et al.*,2010; Bohme *et al.*, 2011;Fernandenz *et al.*, 2010) It can also detects biogenic amine producing bacteria which causes food poisoning (Croxatto *et al.*, 2012).

Direct detection of pathogens

The application of MALDI-TOF MS is very important in rapid identification of microorganism from blood culture in blood stream infections. The preparation of pellets from positive blood cultures can be done by differential centrifugation step to remove blood cells and a washing step to remove nonbacterial components can allow identification of microorganism in less than 1 hour (Croxatto *et al.*, 2012). The early detection of pathogens may be life saving for the patient sometimes. Recent studies have reported that correct identification of pathogen from blood culture bottle by MALDI-TOF can be done in >80% cases. The results varied because of different protocol used for pellet preparation and the type of organism present in blood. Prod'hom *et al.*. have used ammonium chloride as lysing agent and reported that 89% of Gram negative bacteria and 73% of Gram positive bacteria (90% for Staphylococci and 33% of Streptococci) were detected correctly up to the species level by MALDI-TOF MS (Prod'hom *et al.*, 2010) Urine may be tested directly by MALDI-TOF MS due to the high number of bacteria in significant bacteruria. Microorganisms can be detected from urine by including two centrifugation steps by removing leucocytes and the other to collect bacteria (Szabados *et al.*, 2011)

Limitations of MALDI-TOF MS: The initial cost of the instrument MALDI-TOF MS is very high, though the running cost is not too much. The maintenance of the instrument is very important. Laboratory errors may occur which must be monitored. Regular calibration must be done. The quality control strains have to be run. The reference database is still in infancy. In direct detection of microorganism the close relatedness of different species especially with Streptococcus may be responsible for difficulty while analyzing results of MALDI-TOF MS. Gram positive bacterial cell wall also resist lysis. The capsulated bacteria e.g. Streptococcus pneumoniae, Haemophilus influenzae, Klebsiella pneumoniae etc. are responsible for variable results. The major limitation of MALDI-TOF MS in direct detection of microorganism from blood culture was reported in case of mixed bloodstream infection.[76] Similarly, MALDI-TOF MS cannot reliably identify polymicrobial infection in urine (Wang *et al.*, 2013). The identification of fungi in blood culture is poorly done by MALDI-TOF MS (Croxatto *et al.*, 2012).

A major limitation is that antimicrobial susceptibility report is not provided by MALDI-TOF MS. Tiny or mucoid colonies may not be detected by MALDI-TOF MS and can be more rapidly detected by 16S rRNA gene sequencing (Patel, 2015).

Future perspectives of MALDI-TOF MS: Like blood and urine , direct detection of microorganism from other body fluids especially CSF should be developed. The gradual improvement of database is needed. The ability to resolve poly-microbial specimens have to be developed. The detection of antimicrobial susceptibility is also required. Along with detection of pathogens, the other areas where MALDI-TOF MS can be used in future are cancer typing directly from serum, tissue extracts or from body fluids, biomarkers for cancer typing, quantification of peptides etc .(Marvin, 2016). Mutters *et al.*. have reported that early growth detection by digital imaging along with MALDI-TOF MS results will help in rapid detection of microorganism (Mutters *et al.*, 2014). In near future MALDI-TOF MS will play an important role in Microbiology teaching and technologies and over all development of Clinical Microbiolog (Patel, 2015).

Conclusion

To conclude, MALDI-TOF MS has revolutionized the detection of pathogens in Clinical Microbiology Laboratory. It is also important for identification of bacteria that are anaerobic, slow growing and fastidious. It gives rapid and accurate results and intra- laboratory reproducibility is high if the protocols are followed properly. With the improvement of technology and database MALDI-TOF MS will be an essential tool for increasing laboratory efficiency.

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