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MALDI-Tof Assisted Rapid Identification Method for Bacterial Strains

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The Study aimed to compare the efficacy of MALDI-Tof identification system with conventional biochemical tests. Six water samples collected in duplex from water bodies of Mathura region were subjected to culturing, predominant strains isolation. Cultures were subjected to thirty-three different biochemical tests and MALDI-Tof-MS analysis. Biochemical tests predicted that Sample A, B & F belonged to *Achromobacter* genera, Sample D & E to *Rhizobium* genera while Sample C to *Pantoea* Spp. MALDI-Tof analysis demonstrated that Sample A spectra resembled *Achromobacter denitrificans* DSM 30026T DSM, Sample B to *Hafnia alvei* 20740_1CHB, Sample C to *Pantoea* Spp., Sample D & E to *Rhizobium radiobacter* B177 UFL & *Rhizobium radiobacter* DSM 30147T HAM, while Sample F to *Sphinogobacterium mizutaii* DSM 11724T HAM. Both methods displayed 66.67% result similarity. MALDI effectively distinguished between serovars of *Rhizobium*, indistinguishable within biochemical tests. Controversial results were obtained for Sample B & F due to several limitations of Biochemical assays. Identified microbial strains were found associated with opportunistic infections (*Achromobacter* caused), diarrhoea (*Hafnia* caused), neonatal septicemia, death (*Pantoea* spp. caused) and hematological malignancy (*Rhizobium radiobacter* caused) indicating the etiology of several endemic diseases. Thus, results indicated that MALDI could develop as a rapid identification tool over conventional biochemical assays.

Keywords: MALDI-Tof-MS; Biochemical analysis; *Achromobacter*; *Hafnia*; *Pantoea* and *Rhizobium*.

Pathogenic microbial infection is the leading killer of human being and animals throughout the world and has affected the economy, productivity of the country limiting the amount of livestock's[1, 2, 3]. Although India ranks among the largest livestock populated county yet ranks too low in productivity as compared to others due to the prevalence of infectious diseases affecting productivity and reproductive potential of animals[4, 5]. Rapid diagnosis is the key to success in combating against pathogenic microbial infections[6]. Although host immune system provides a strong defense against these ubiquitous killers yet these microbes have evolved mechanisms to dodge host immune responses. Moreover the delay in clinical identification of microbes have provided a window of opportunity to such pathogens leading to the establishment of a disease[3, 7].

The development of modern medical sciences has fueled the evolution from conventional microbial identification methods i.e., morphological identification, cultural characteristics, biochemical, staining, immunological assays to the most recent evolved method of the 20th Century the 16 S rRNA sequencing but each of these confers some advantages over the other so are alternatively

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used[6]. However, none of the above methods has the ability to identify microorganisms within minutes without compromising with the fidelity of the results[8].

MALDI-MS (Matrix assisted laser desorption ionization mass spectroscopy) refers to a rapid, versatile, in vitro technique for molecules identification based on photo-desorption of a sample embedded within a light absorbing matrix[9]. A pictorial representation of MALDI-Tof MS is depicted in the fig. 1. Although classical MALDI measured m/z ratio only in the range of 10 to 50 Da, being insignificant for microbial analysis but in the 21st century the path-breaking discovery of soft ionization method (eg.: Electrospray ionization) by John B. Fenn and Koichi Tanaka who won 2002 Nobel Prize in Chemistry extended the boundaries of this technology into new domains paving the way towards microbial identification[6, 10]. Soft ionization technology has enabled macromolecular analysis without fragmentation. MALDI assisted microbial identification began in relation to Mars mission and aimed to analyze its soil samples[11, 12].

Thus spectra analysis of novel biomarkers worked out as a fingerprint technology in identification of microorganisms through mass spectroscopy (MS)[13]. As proteins are the most abundant (50-70%), highly diverse yet conserved biomolecule present within a cell so is selectively used as biomarkers in MS spectra analysis[6, 13].Since ancient time scientists, researchers and medical practitioners have speculated to develop a rapid, reliable and effective method for identification of microbial samples from biological and non-biological sources. Classical methods of microbial identification are based on the morphological or biochemical analysis which usually takes several days to months and requires expertise in the field[13, 15]. Early diagnosis of a particular pathogen could not only alarm us of the root cause of a particular disease, but could also provide an insight into the reservoirs of these pathogenic microbes hence lending us a golden opportunity to eliminate these pathogenic microbes. The work aims to develop a rapid, versatile, in vitro method for pathogenic microbial identification from local water samples within minutes and compare its efficacy with that of standard biochemical tests already used.

MATERIALS AND METHODS

Collection and Isolation of Microbial samples

Six different samples collected from different water bodies of Mathura region were analyzed for microbial contaminants by repeated subculturing over nutrient agar medium in triplets. Incubation of 24-48 hrs at 37°C was provided and microbial growth analyzed. The most prominent microbial colonies from each culture were selectively amplified and obtained in their pure forms through repeated subculturing, streaking and U.V light illumination. The plan of work was to simultaneously perform a comparative study of microbial sample analysis through Biochemical and MALDI-Tof analytical methods as depicted in the fig. 2.

Biochemical identification of Microbial samples

The aim of the Biochemical characterization of microbes was to categorize microbes on the basis of their ability to synthesize

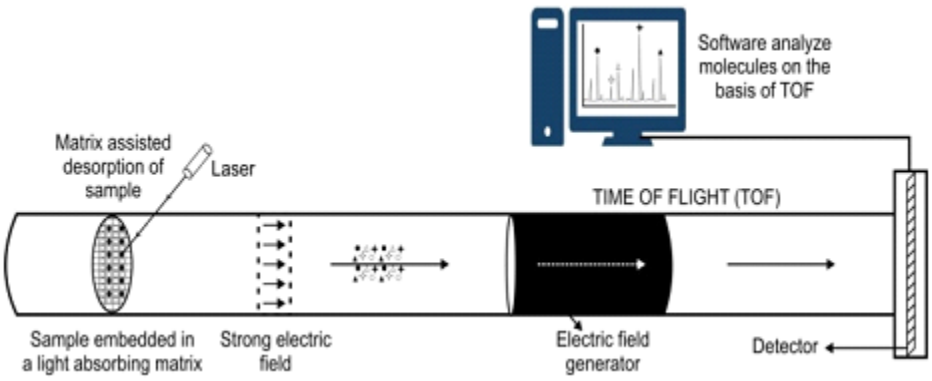


Fig. 1. Principle of MALDI-Tof: Light-absorbing matrix assisted desorption of analyte molecules causing their ionization to form protonated molecules whose time of flight was measured with the help of a detector.

specific enzymes which will proffer it a selective advantage over a particular substrate and to carry out a specific biochemical reactions. Thus, microbes were classified on the basis of their ability to grow on different growth media, their ability to ferment sugars, gas production, their nature aerobic or anaerobic, etc.

Thirty-three different biochemical tests were performed to evaluate the nature of six isolated microorganisms; results obtained were comparatively analyzed with reference standards predicting the nature of tested samples.

MALDI-Tof based microbial identification

The prerequisite for MALDI-Tof analysis of a microbial sample was the requirement of uncontaminated microbial cultures, as any protein contaminants may effectively vary the results significantly so involved a pretreatment of the sample before analysis. The type of pretreatment involved depended upon the nature of the sample i.e., on bacterial types (Gram '+'or '-'). As the thick peptidoglycan cell wall (70-80 nm) of gram positive bacteria may interfere with cell lysis and protein isolation so a physical (Ultrasonic) or enzymatic (lysozyme-mediated) pretreatment was required before analysis[16].

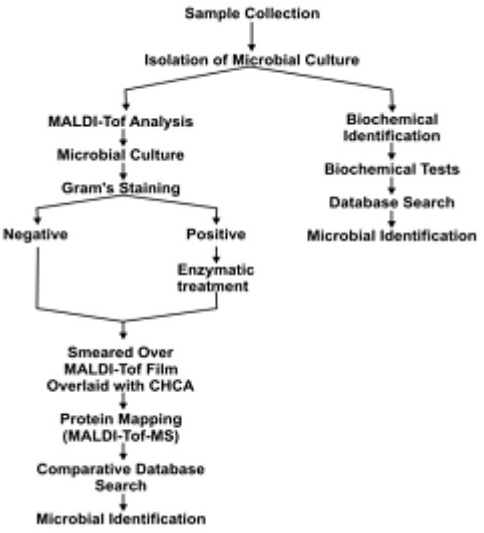


Fig. 2. Flowchart of the work performed including biochemical and MALDI-Tof characterization.

Sample Preparation & Experimental Procedure for MALDI-Tof

Sample preparation was performed according to the method as prorogated by Bruyne *et al.*, 2011[17]. All six samples of pure uncontaminated cultures (Grams '+' pretreated & Grams '-' untreated) isolated and maintained over agar plates were picked with sterile tips, washed with HPLC grade water and centrifuged. 70% of formic acid and acetonitrile (ACN) were added in a 1:1 ratio to bacterial palate and vortexed for 30 sec[18]. Supernatant thus obtained was smeared over thin film within different chambers present over a steel MALDI plate overlaid with CHCA (α-Cyano-4-dihydroxybenzoic acid) in a ratio of 50:48:2 acetonitrile: water: trifluoroacetic acid solution[15, 19, 20]. The matrix used in MALDI-Tof analysis is depicted in table 1. Smear was air-dried before analysis and each sample were spotted twice to check the reproducibility of the result.

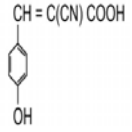
RESULTS AND DISCUSSION

The experimental results of biochemical analysis are summarized in table 2 while results of microbial analysis through MALDI-Tof are represented in fig 3.

Results of Biochemical Assays

Gram's staining indicated that all six isolated strains were found to be gram negative in nature indicating their pathogenic potential and probable antimicrobial drug resistance as found in major gram negative microbes[21, 22, 23]. Biochemical tests were performed to determine the ability of these microbial strains to metabolize varied carbon sources such as Carbohydrates (Adonitol, Arabitol, Cellobiose, Glucose, Maltose, Mannitol, Mannose, Palatinose, Sucrose, Tagatose, Trehalose); Carbohydrates derivatives (Sorbitol, Citrate); Lipids (Lipases) & nitrogenous wastes such as urea (Urease-producing). Microbes were also categorized on the basis of their capability to biosynthesize specific enzymes (Pyrrolydonyl-Arylamidase, Acetyl Glucosaminidase, Glutamyl transferase, Glucosidase, Xylosidase, Glutamyl, Proline & Tyrosine arylamidase, Lactate & Succinate alkalinisation, etc), to resist antimicrobial agents such as 2,4 Diamino-6,7-diisopropyl-pteridine (O/129) or to assimilate other metabolites (Malate/ Lactate), etc as represented in table 2.

Table 1. Matrix used while performing MALDI-Tof mass spectroscopy

Matrix Used	Structure	Molecules
CHCA α-Cyano-4-hydroxy Cinnamic acid		Peptides/ Proteins

Thus, the biochemical tests aimed to categorize microbes on the basis of their ability to synthesize specific enzymes which will proffer it a selective advantage to use a particular substrate or to carry out specific biochemical reactions.

Results of the biochemical study were restricted to microbial genera and were not species specific. Biochemical investigation predicted that Sample A, B & F belonged to *Achromobacter*

Table 2. Results of Biochemical tests performed for microbial identification where '+' represents positive results while '-' represents negative results of the concerned test involved

S. No.	Staining/ Biochemical tests performed	Amount /Well	Sample A	Sample B	Sample C	Sample D	Sample E	Sample F
1.	Adonitol *(ADO)*	0.1875 mg	-	-	-	+	+	-
2.	L-Pyrrolidonyl-Arylamidase (*PyrA*)	0.018 mg	+	+	+	+	+	+
3.	L-Arabitol *(IARL)*	0.3 mg	-	-	-	+	+	-
4.	D-Cellobiose *(dCEL)*	0.3 mg	-	-	+	-	-	-
5.	β-N-acetyl-glucosaminidase *(BNAG)*	0.0408 mg	-	-	-	+	-	-
6.	Glutamyl Arylamidase pNA *(AGLTp)*	0.0324 mg	+	+	-	-	-	+
7.	D-Glucose *(D-GLU)*	0.3 mg	-	-	+	-	-	-
8.	γ-Glutamyl-transferase *(GGT)*	0.0228 mg	-	-	-	-	-	-
9.	β-Glucosidase *(BGLU)*	0.036 mg	-	-	+	-	-	-
10.	D-Maltose *(dMAL)*	0.3 mg	-	-	+	-	-	-
11.	D-Mannitol *(dMAN)*	0.1875 mg	-	-	+	-	-	-
12.	D-Mannose *(dMNE)*	0.3 mg	-	-	+	-	-	-
13.	β-Xylosidase *(BXYL)*	0.0324 mg	-	-	+	-	-	-
14.	L-ProlineArylamidase *(ProA)*	0.0234 mg	+	+	-	+	+	+
15.	Lipase *(LIP)*	0.0192 mg	-	-	-	-	-	-
16.	Palatinose *(PLE)*	0.3 mg	-	-	+	-	-	-
17.	Tyrosine Arylamidase *(TyrA)*	0.0276 mg	-	-	-	+	+	-
18.	Urease *(URE)*	0.15 mg	-	-	-	+	+	-
19.	D-Sorbitol *(dSOR)*	0.1875 mg	-	-	+	-	-	-
20.	Saccharose/ Sucrose *(SAC)*	0.3 mg	-	-	+	-	-	-
21.	D- Tagatose *(dTAG)*	0.3 mg	-	-	-	-	-	-
22.	D-Trehalose *(dTRE)*	0.3 mg	-	-	+	-	-	-
23.	Citrate *(CIT)*	0.054 mg	+	+	-	-	-	+
24.	Malonate *(MNT)*	0.15 mg	-	-	-	-	-	-
25.	L-lactate alkalinisation *(ILATK)*	0.15 mg	+	+	-	-	-	+
26.	α-Glucosidase *(AGLU)*	0.036 mg	-	-	-	+	+	-
27.	Succinate alkalinisation *(SUCT)*	0.15 mg	+	+	-	-	-	+
28.	βN-acetyl-galactosaminidase *(NAGA)*	0.0306 mg	-	-	-	-	-	-
29.	α-Galactosidase *(AGAL)*	0.036 mg	-	-	-	+	-	-
30.	Coumarate *(CMT)*	0.126 mg	-	-	-	-	-	-
31.	O/ 129 Resistance *(O129R)*	0.0105 mg	-	-	-	-	-	-
32.	L-Malate assimilation *(IMLTa)*	0.042 mg	-	-	-	-	-	-
33.	L-lactate assimilation *(ILATa)*	0.186 mg	-	-	-	-	-	-
	Preliminary Results of Biochemical analysis		*Achromobacter*	*Achromobacter*	*Pantoea Spp.*	*Rhizobium*	*Rhizobium*	*Achromobacter*

genera, Sample C to *Pantoea*, while Sample D & E to *Rhizobium* genera.

It was also noted that among the thirty three different biochemical tests performed on Sample D & E, βN-acetyl-galactosaminidase (NAGA) and α-Galactosidase (AGAL) activity were found to be significantly different indicating that Sample D & E belonged to different serovars of *Rhizobium* genera.

Results of MALDI-Tof analysis

MALDI-Tof analysis of the microbial sample was based on ribosomal protein desorption, as these are highly conserved yet unique in sequence within each and every living organism so could easily correspond to a particular species, strain or serovar of a microbe. Spectra obtained were compared with standard spectra present within the reference and with an identification matrix, a hit score was generated. If the spectra match score was found to be greater than the threshold value, the match was declared to be significant (For Bruker Biotype software Score value greater than or equal to 1.7) else not[6]. On the basis of score value genera, species, strains or serovars were identified.

Sample A, B, C, D, E & F of microbial strains were analyzed on the basis of their MALDI spectra as shown in fig 3 and were found to be *Achromobacter denitrificans* DSM 30026T DSM; *Hafnia alvei* 20740_1 CHB; *Pantoea* spp.; *Rhizobium radiobacter* B177 UFL, *Rhizobium radiobacter* DSM 30147T HAM and *Spingobacterium mizutaii* DSM 11724T HAM. Although MALDI-Tof provided reliable results in almost all samples yet Sample C analysis was limited to only species level due to the limitation of Biotyper reference library.

DISCUSSION

Comparative analysis of Biochemical and MALDI-Tof displayed 66.67 % result similarity. In Sample B & F controversial results were obtained, biochemical tests indicated that Sample B & F belonged to *Achromobacter* while MALDI-Tof indicated that Sample B was *Hafnia alvei* 20740_1 CHB while Sample F was *Sphingobacterium mizutaii* DSM 11724T HAM as shown in fig 3. Subsequent, 16 S rRNA sequencing has supported MALDI-Tof results over biochemical assays reflecting several limitations of biochemical tests. Sample D & E were indistinguishable in biochemical assays, yet MALDI-Tof adequately differentiated in between the two, indicating that although Sample D & E belonged to the Genus *Rhizobium* yet were its different serovars, *Rhizobium radiobacter* B177 UFL & *Rhizobium radiobacter* DSM 30147T HAM as indicated by their MALDI spectra provided in Fig. 3.

The isolated and identified microbial contaminants such as *Hafnia alvei* has been documented to enter into the local water bodies through contaminated fecal discharge, while *Achromobacter denitrificans*, *Pantoea* spp and *Rhizobium* are ubiquitous soil bacterium, *Sphingobacterium mizutaii* has been associated with activated sludge[24, 25, 26, 27, 28].

Majority of the isolated microbes were opportunistic pathogens infecting animals, humans especially children and immune-compromised individuals causing pneumonia (*Achromobacter denitrificans*), severe diarrhoea (*Hafnia alvei*), hematological malignancy, neonatal sepsis (*Pantoea* Spp.) or carcinoma (*Rhizobium radiobacter*)[24, 25, 27, 29, 30, 31]. As the major isolated microbial strains were found to be pathogenic in nature so study disclosed the etiology of several endemic disease occurring within the region.

Researchers have reported the excessive deaths of bovines in Mathura and adjoining areas due to severe diarrhoea which might be caused by *Hafnia alvei*, reservoirs in the local water bodies of the region[24, 32]. The high rate of hematological malignancy, infections in shepherds might be reasoned to the presence of *Rhizobium* microbial strains in local water bodies and storage utensils especially plastic wares[29].

Documented research publications of reputed institutes have also indicated that almost all isolated microbes displayed potential anti-microbial drug resistance, enhancing their pathological virulence. Parallel research publication has displayed *Achromobacter* to be resistant to sulfa drugs such as Sulfamethoxazole, Sulfonamides, aminoglycosides, etc, while both *Hafnia* & *Pantoea* species are predicted to be multi drug resistant[24, 25, 31, 33]. Almost no information has been reported for the pathogenicity or drug resistance of *Sphingobacterium mizutaii* strains.

Limitations of Mass Spectroscopy

The sensitivity of MALDI poses the greatest challenges as a slightest contaminant can vary its results so precautions should be taken to either obtain a pure microbial culture or to obtain a specific biomarker protein before analysis. Moreover only bacterial strains possessing a potential protein biomarker can be detected and

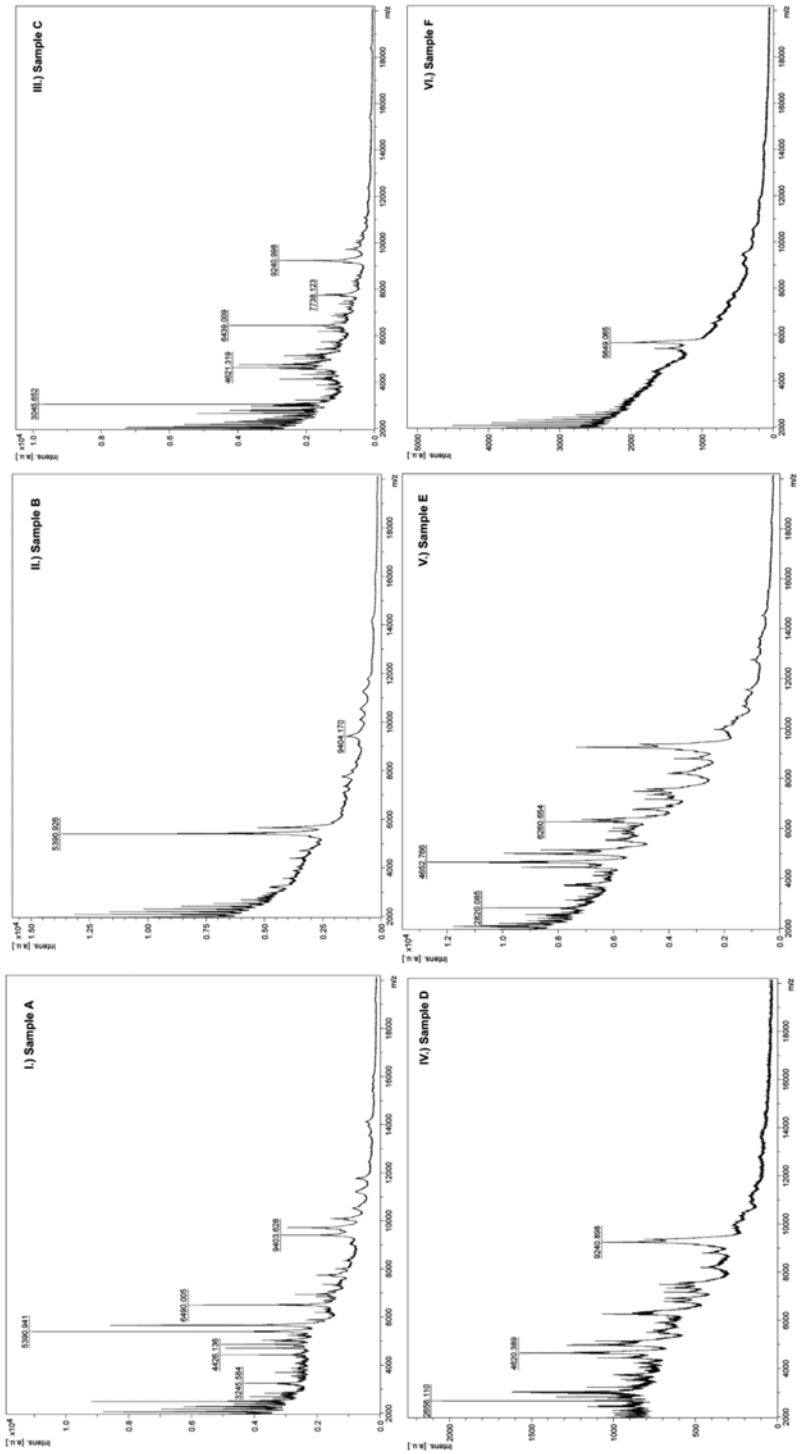


Fig. 3. Mass Spectroscopic (MALDI-TOF) analysis of the isolated bacterial samples from local water bodies. a.) Sample A, analysis exhibited resemblance to Achrobacter denitrificans DSM 30026T DSM, b.) Sample B, analysis exhibited resemblance to Hafnia alvei 20740_1 CHB, c.) Sample C, analysis exhibited resemblance with *Pantoea* spp, d.) Sample D, analysis exhibited resemblance to *Rhizobium radiobacter* B177 UFL e.) Sample E, analysis exhibited resemblance to *Rhizobium radiobacter* DSM 30147T HAM f.) Sample F, analysis exhibited resemblance to *Sphingobacterium mizutaii* DSM 11724T HAM.

compared with that of standard reference strains whose spectra are available while novel isolated strains cannot be analyzed until their spectra becomes available in standard MALDI-Tof library[20, 34, 35, 36]. Continuous updating of the database can reduce the risk of any mismatch taking the error rate close to zero and the identification rate close to perfection[18, 37].

The analysis of MS is also limited to only 10^4 CFU/ml of sample, bacterial concentration lower then that cannot be detected through MS spectroscopy[3, 19]. Although MALDI mass spectroscopy is established for pure cultures but the use of this technique for analysis of direct biological samples is still a challenge as host proteins, enzymes may interfere with its spectra[38]. So, for practical applications to biological samples a pretreatment is involved which could specifically separate the protein biomarker before analysis and identification.

CONCLUSION

As early diagnosis may trigger early treatment thus enhances patient survival rate, reduces complications and decreases expenses on medicines, so the work provides a light on the development of rapid diagnosis method through MALDI MS analysis which could be path breaking in the field of clinical diagnosis reducing diagnosis time from months to minutes. Results clearly stated that MALDI proved a better option providing significant results with strain level specifications as compared to biochemical tests which were able to identify only the genus of the microbes with limited accountability. Spectra analysis through MALDI is completed as soon as sample is uploaded and the hit score can predict about the significance of the result obtained making MALDI cost and time effective. Results even indicated that MALDI can also be used in routing health checkups in patients with pathogenic bacterial infections to detect for the efficacy of the treatment provided and for the development of the MDR strains. It indicated that MALDI-Tof could act as a jack of all trades and help us to win the war against pathogenic microbes. With the scope of automation MALDI is deemed to be the method of choice in the fight against pathogenic microbes during 21st Century.

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