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The Role of Matrix-Assisted Laser Desorption Ionization-Time of Flight- Mass Spectrometry (MALDI-TOF-MS) in the Identification of Non-fermenting Gram-Negative Bacilli from Clinical Isolates in a Tertiary Care Hospital

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Abstract

Non-fermenting Gram-negative bacilli (NFGNB) are a heterogeneous group of aerobic non-spore forming bacilli. Matrix-Assisted Laser Desorption Ionization-Time of Flight- Mass Spectrometry (MALDI-TOF-MS) is a quick, precise diagnostic method that could identify the isolates at the species level. The aim of the study is to identify the non-fermenters isolated from various clinical infections by MALDI-TOF analysis and to detect the antibiogram of these isolates. The non-fermenters were isolated from various clinical specimens, plated on Blood agar and MacConkey agar and incubated at 37ºC for 24 hours. Identification was done on the basis of colony morphology, Gram stain, and MALDI-TOF Analysis. Antimicrobial Susceptibility Testing was done by Kirby- Bauer Disc Diffusion Technique using commercially available antibiotics on Muller-Hinton Agar. A total of 360 samples were studied. The majority of the non-fermenters were from Blood/ Body fluids (39.72%). Acinetobacter spp (31.67%) was the most common isolate, followed by Pseudomonas spp (22.50%). Other non-fermenters like Stenotrophomonas maltophilia, Burkholderia spp, Elizabethkingia spp, Achromobacter spp, Chryseobacterium spp, Ralstonia spp, Pandoraea spp, Rhizobium radiobacter, Sphingomonas spp, Ochrobactrum spp, Myroides odoratimimus and Brevundimonas spp were rarely isolated non-fermenters. Most of the strains were multi drug resistant to commonly used antibiotics like Cephalosporins, Aminoglycosides and Carbapenems. Our results showed that identifying non-fermenters using MALDI-TOF MS is a reliable and rapid technique that shortens the time to initiate appropriate therapy and even the length of the hospital stay.

Keywords: Non-fermenters, Maldi-Tof, Multidrug resistant

Introduction

Non-fermenting Gram-negative bacilli (NFGNB) are a heterogeneous group of aerobic non-spore forming bacilli. They do not use carbohydrates as a source of energy and instead break them down through

oxidative metabolic pathways [1]. They occur as saprophytes in the environment; some are commensals in the human gut [2] and act as multi drug resistant opportunistic pathogens in hospital infections [3]. These organisms proliferate on standard culture media, but definitive identification can be difficult because most of the species are relatively inert in the conventional biochemical tests [4]. Gram-negative non-fermenters are usually a niche pathogen that cause opportunistic infections in critically ill or immunocompromised patients [5]. NFGNB are ubiquitous and are isolated from various hospital instruments and the body surfaces of healthcare workers [6]. They can cause numerous infections in hospitalized patients, accounting for approximately 15% of all Gram-negative bacterial infections [7, 8]. This group includes several genera such as Acinetobacter, Pseudomonas, Stenotrophomonas, Burkholderia and other some less frequently isolated genera like Achromobacter, Ochrobactrum, Flavobacterium, Chryseobacterium, Elizabethkingia, Sphingobacterium and Ralstonia [9, 10]. These organisms are not routinely identified because some of them are slow growers and require special culture media for isolation. The most prevalent pathogens in patients are Pseudomonas aeruginosa, Burkholderia cepacia and Stenotrophomonas maltophilia [11, 12]. Because of their low biochemical reactivity these bacteria are often difficult to identify using conventional microbiology methods [13]. The extensive use of antibiotics in healthcare has made NFGNB a significant pathogen in recent years [14]. They have been detected in septicemia, meningitis, pneumonia, urinary tract infections, ventilator-associated pneumonia, wound infections, osteomyelitis and surgical site infections [15].

Antibiotic resistance in NFGNB is primarily due to antimicrobial inactivating enzymes that prevent access to bacterial targets and point mutations that alter target structures or cellular functions [16, 17]. Metallo-β- lactamases and extended spectrum β -lactamases are produced by NFGNB, which are inherently resistant to a wide range of antibiotics. Gram-negative non-fermenters are increasingly showing signs of multidrug resistance. Several strains have now been identified that are resistant to all commonly used antibiotics [18, 19].

The precise clinical significance of these isolates is still being determined, and routine identification of non-fermenters is challenging, leading to the misidentification of these pathogens [20]. Molecular methods are expensive and labor-intensive and automated systems are not very selective. Therefore, rapid and reliable technology is the need of the hour [21,22]. In clinical Microbiology, Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) has become a more useful technique for species identification [23]. Identification of non-fermenters has become easy with the introduction of MALDI-TOF-MS [24]. This simple technique replaces traditional methods for identifying microorganisms in the clinical laboratory and can overcome many challenges in identifying bacteria and fungi. In many cases, this reduces the time to initiate appropriate therapy and even the length of the hospitalization [25-27].

The present study aimed to identify the isolated NFGNB from different clinical infections using MALDI-TOF analysis and to determine the antibiogram of these isolates.

Methodology

This study was conducted in the laboratory of the Microbiology Research Center over six months. This study includes clinical specimens such as urine, pus, respiratory, tip, blood and body fluid. All clinical samples, regardless of age or gender, were sent in sterile containers from the central Microbiology laboratory to the Microbiology Research Center at Father Muller Medical College Hospital in Mangalore. These samples were incubated for 24 hours at 37°C after being inoculated on blood agar and MacConkey

agar. After the incubation under aerobic conditions, the identification of the isolates was done on the basis of colony morphology, Gram stain and MALDI-TOF Analysis. The identification using MALDI- TOF MS was performed by the extended direct transfer method with a Microflex LT instrument using FlexControl 3.4 and MALDI BioTyper software (Bruker Daltonik, Germany, Version 4.1.100). The names of the best matches in the database were recorded and only scores greater than 1.7 were taken into consideration for identification.

The susceptibility of bacterial isolates against different antibiotics was determined by the Kirby-Bauer Disc Diffusion Technique. The Clinical and Laboratory Standards Institute (CLSI) guidelines were followed in the interpretation of the results.

Work began after receiving approval from the Institutional ethics committee at FMIEC, Mangalore **(**Ref. No: FMIEC/ CCM/743/2022).

MALDI-TOF Extended Direct Transfer (eDT) Method: After colonies appear on the plate MALDI-TOF was performed for species-level identification. Briefly, an isolated fresh colony was smeared as a thin film directly onto an empty position on the MALDI target plate and overlaid the material with 1.0µL of 70% formic acid. After drying at room temperature, the smear was overlaid with 1.0µL of matrix solution, α-Cyano-4-hydroxycinnamic acid (HCCA) and dried at room temperature. After entering the patient data, the sample plate was placed in the MALDI-TOF device. The MALDI-TOF software automatically processed the data by analysing the spectra generated from the cumulative laser shots. Subsequently, the obtained spectra were compared with reference libraries for bacterial identification, aiming to find the most accurate match.

Results

In this study, 360 non-fermenters were analyzed by MALDI-TOF MS, including different species **(Table 1).** Acinetobacter spp was the most common isolate, accounting for 114 (31.67%), followed by Pseudomonas spp 81 (22.50%), Stenotrophomonas maltophilia 56 (15.56%) and Burkholderia spp 32 (8.89%). Rare isolates such as Elizabethkingia spp, Achromobacter spp, Chryseobacterium spp, Ralstonia spp, Pandoraea spp, Rhizobium radiobacter, Sphingomonas spp, Ochrobactrum spp, Myroides odoratimimus, Brevundimonas spp were obtained, together accounting for 21.38% (77) of the isolates. Among the total 360 non-fermenters**,** 60% (n=216) were isolated from male and 40% (n=144) were from female patients, with male to female ratio 1.5:1. The maximum number of isolates, 201 (55.83%) were obtained from the adults (21-60 age) followed by Geriatric population 124 (34.44%) and Paediatric group 35 (9.72%). The demographic details of the samples were shown in **Table 2.**

Figure 1 depicts the distribution of organisms in the various clinical samples. Most non-fermenters were isolated from Blood and body fluids143 (39.72%) and respiratory specimens 82 (22.78%). Of the isolates, 114 (31.66%) were Acinetobacter spp, 81 (22.5%) were Pseudomonas spp and 56 (15.5%) were Stenotrophomonas maltophilia.

The clinical conditions in which non-fermenters were isolated in our study include Respiratory tract infections (21.67%), Blood stream infection (18.33%), Urinary tract infections (14.72%), Pyogenic infections (13.06%), Bone and joint infection (4.17%), Skin and soft tissue infections (3.06%).

The antibiogram of the isolates is shown in **Table 3**. Most of the isolates were resistant to common antibiotics such as cephalosporins, aminoglycosides and carbapenems. Acinetobacter spp exhibited a very high (93.46%) resistance to cefotaxime (30 µg), which was followed by a 91.59% resistance to

carbapenem [imipenem (10µg) and meropenem (10 µg)]. Ceftazidime (30 µg) was resistant to Chryseobacterium spp. (100%) and Pseudomonas spp. (71.42%). Cephalosporins showed a high resistance rate against most Gram-negative bacteria.

Table 1: Distribution of non-fermenters in clinical specimens (n=360)

Bacteria	No of Isolates (n)	Percentage (%)
Acinetobacter species (A. baumannii, A. guillouiae, A. pitti, A. bereziniae, A. nosocomialis, A. junii, A. towneri, A. schindleri, A. radioresistens, A. soli, A. variabilis, A. ursingii, A. indicus, A. venetianus)	114	31.67
Pseudomonas species (P. aeruginosa, P. oxyzihabitans, P. monteilii, P. stutzeri, P. guariconensis, P. mendocina, P. putida, P. mosselii, P. fulva, P. otitidis)	81	22.50
Stenotrophomonas maltophilia	56	15.56
Burkholderia species (B. cepacia, B. cenocepacia, B. multivorans, B. thailandensis, B. ambifaria, B. mallei, B. pseudomallei, B. pyrrocinia, B. diffusa, B. gladioli, B. veitnamiensis)	32	8.89
Elizabethkingia species (E. anophelis, E. meningoseptica, E. miricola)	19	5.28
Achromobacter species (A. xylosoxidans, A. dentrificans, A. mucicolens)	16	4.44
Chryseobacterium species (C. indologenes, C. gleum, C. anthrosphaerae, C. aquifrigidense, C. shandongense)	13	3.61
Ralstonia species (R. pickettii, R. mannitolilytica)	7	1.94
Pandoraea species (P. apista, P. sputorum, P. pnomenusa)	5	1.39
Rhizobium radiobacter	4	1.11
Sphingomonas species (S. paucimobilis, S. sanguinis, S. pseudosanguinis)	4	1.11
Ochrobactrum species (O. anthropi, O. intermedium)	3	0.83
Myroides odoratimimus	3	0.83
Brevundimonas species (B. diminuta, B. aurantica)	3	0.83
TOTAL	**360**	**100**

Table 2: Demographic details of the clinical specimens (n=360)

VARIABLES	SEX		AGE (YEARS)		
	Male	Female	Pediatric (1-20)	Adult (21-60)	Geriatric (>60)
Acinetobacter spp	67	48	14	61	40
Pseudomonas spp	46	36	3	55	24
Stenotrophomonas maltophilia	33	22	1	29	25
Burkholderia spp	23	9	3	17	12
Elizabethkingia spp	15	4	3	11	5
Achromobacter spp	10	4	2	10	2
Chryseobacterium spp	4	9	2	4	7
Ralstonia spp	6	1	1	4	2
Pandoraea spp	2	3	1	1	3
Rhizobium spp	1	3	3	1	0
Sphingomonas spp	4	0	0	3	1
Ochrobactrum spp	2	1	0	2	1
Myroides spp	2	1	0	3	0
Brevundimonas spp	1	3	2	0	2
Total	**216**	**144**	**35**	**201**	**124**

Table 3: Percentage of Antimicrobial resistant pattern of non-fermenters

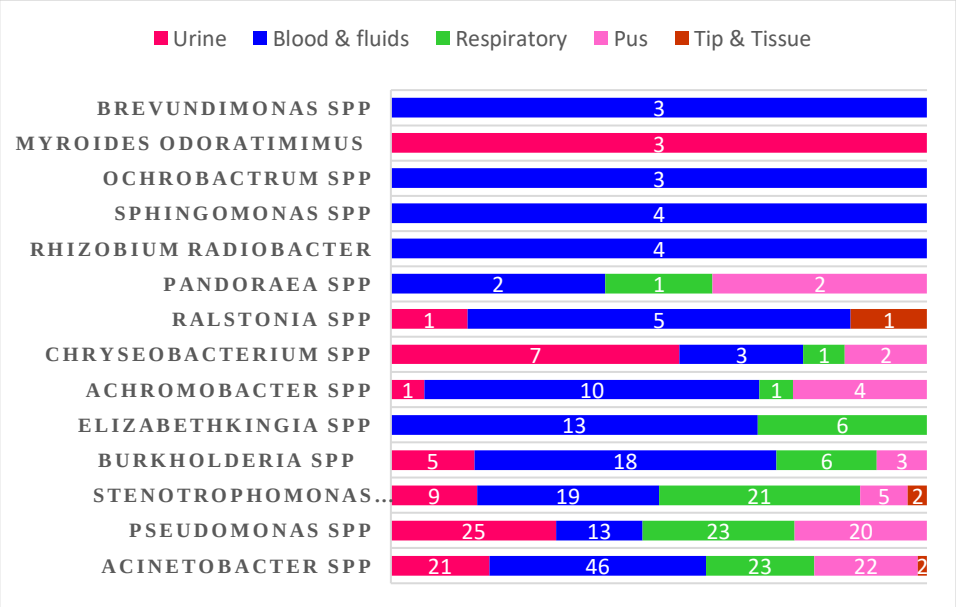
Groups	Antibiotics	*Acineto.* spp n (%)	*Pseudo.* spp n (%)	*Steno. maltophilia* n (%)	*Burkholderia* spp n (%)	*Achromobacter* spp n (%)	*Elizabeth-kingia* spp n (%)	*Chryseobact-eriu m* spp n (%)	TOTAL %
No. Tested	-	116	81	55	32	15	19	13	
Penicillins	-	**R**	**R**	**R**	**R**	**R**	**R**	R	**R**
	AMP (10 µg)	3 (30.14)	3 (3.90)	2 (3.70)	3 (12.50)	0	3 (25.00)	0	15.05%
	AMC (20 µg)	35 (32.71)	0	12 (22.22)	5 (20.83)	4 (28.57)	4 (33.33)	8 (88.89)	32.36%

Cephalosporins	**CXM (30 µg)**	36 (33.64)	0	13 (24.07)	1 (4.17)	9 (64.28)	10 (83.33)	8 (88.89)	42.58%
	CTX (30 µg)	**100 (93.46)**	**55 (71.42)**	40 (74.07)	5 (20.83)	**12 (84.71)**	9 (75.00)	7 (77.78)	**71.03%**
	CAZ (30 µg)	3 (2.80)	**55 (71.42)**	24 (44.44)	1 (4.17)	1 (7.14)	10 (83.33)	**9 (100)**	44.17%
Floroquinolones	**CIP (5 mcg)**	21 (19.63)	10 (12.99)	9 (16.67)	0	4 (28.57)	0	3 (33.33)	15.88%
	LE (5 mcg)	7 (6.54)	12 (15.58)	9 (16.67)	0	1 (7.14)	0	7 (77.78)	15.29%
Sulfamethoxazole-trimethoprim	**SXT (25 mcg)**	87 (81.31)	3 (3.90)	12 (22.22)	1 (4.17)	1 (7.14)	0	0	16.96%
Aminoglycosides	**AK (30 mcg)**	70 (65.42)	35 (45.45)	48 (88.89)	13 (54.17)	4 (28.57)	11 (91.67)	8 (88.89)	66.15%
	G (10 µg)	84 (78.50)	17 (22.08)	48 (88.89)	14 (58.33)	4 (28.57)	**12 (100)**	8 (88.89)	66.46%
Carbapenems	**IPM (10 mcg)**	98 (91.59)	34 (44.16)	40 (74.07)	3 (12.50)	1 (7.14)	11 (91.67)	8 (88.89)	58.57%
	MRP (10 µg)	98 (91.59)	33 (42.86)	40 (74.07)	2 (8.33)	1 (7.14)	11 (91.67)	8 (88.89)	57.79%

β Lactamase inhibitors	**PIT (100 mcg)**	72 (67.29)	37 (48.05)	**49 (90.74)**	6 (25.00)	1 (7.14)	3 (25)	8 (88.89)	50.30%
	CFS (30 mcg)	86 (80.37)	35 (45.45)	47 (87.04)	5 (20.83)	1 (7.14)	0	8 (88.89)	47.10%
Polypeptides	**PB (300 units)**	0	0	9 (16.67)	**15 (62.50)**	0	6 (50)	2 (22.22)	21.62%

AMP-Ampicillin, AMC-Amoxicillin-Clavulanic acid, CXM-Cefuroxime, CTX- Cefotaxime, CAZ-Ceftazidime, CIP-Ciprofloxacin, LE-Levofloxacin, SXT- Trimethoprim –sulfamethoxazole, AMK-Amikacin, G-Gentamycin, PB- Polymyxin B, IMP-Imipenem, MRP-Meropenem, CFS-Cefoperazone/Sulbactam, PIT- Piperacillin/ Tazobactam

Figure 1: Distribution of organisms in different Clinical samples



Discussion

Non-fermenters are opportunistic pathogens known to cause hospital-acquired infections. The indiscriminate and widespread use of antibiotics has led to an increase in multidrug-resistant organisms and an increase in hospitalization length, mortality, morbidity, and cost.

In our study a total of 360 samples were subjected to characterize the non-fermenters. Among them, 216 were men (60%) and 144 were women (40%). Chaudhary et al. [16] reported a male proportion of 60% and Malini et al. [2] of 68%. Regarding age distribution in this study the highest percentage was in the

adults (56.11%), which is consistent with other studies by Kalidas Rit et al. in Kolkata (72%) and by Rahbar et al. (70%), probably due to substandard immune system [6,11]. Our study shows that majority of non-fermenters were isolated from blood and body fluids 143 (39.72%), followed by respiratory infections 82 (22.78%), which is in accordance with the observations made by Sambyal et al [14].

The most common non-fermenting isolates in the present study were Acinetobacter spp (31.67%) and Pseudomonas spp **(**22.50%**)** which is in accordance with few investigators. Sambyal et al. found Acinetobacter baumannii (44%) followed by Pseudomonas aeruginosa (40%), while Yadav et al. reported high prevalence of Acinetobacter baumannii (64.29%) followed by Pseudomonas aeruginosa (35.71%) [7,14]. Other rare non-fermenters isolated in the present study were Stenotrophomonas maltophilia, Burkholderia spp, Elizabethkingia spp, Achromobacter spp, Chryseobacterium spp, Ralstonia spp, Pandoraea spp, Rhizobium radiobacter, Sphingomonas spp, Ochrobactrum spp, Myroides odoratimimus, Brevundimonas spp. These were very difficult to identify by conventional technique. The introduction of MALDI-TOF MS in the clinical Microbiology laboratory is revolutionizes the diagnosis of infectious disease and clinical care. Identifying species with similar phenotypic, genotypic, and biochemical traits was previously impossible. However, this is now possible with the technological advancements and the growth of databases holding the spectra of known organisms. Identification using MALDI-TOF MS has led to improvements in clinical care by diagnosing infections caused by relatively rare species early and shortening the time to diagnosis. Employing this technique, the isolates in our study could be identified in just 20 minutes after the colonies appeared on the culture media. Early identification of bacteria, which enables rapid initiation of treatment, is the critical application of MALDI-TOF in bacteriology.

In the present study, majority of the strains were multidrug-resistant to commonly used antibiotics such as cephalosporins, aminoglycosides and carbapenems. Cefotaxime resistance in Acinetobacter spp. was very high (93.46%) followed by carbapenem (imipenem and meropenem - 91.59%). Ceftazidime was resistant against Chryseobacterium spp. (100%) and Pseudomonas spp. (71.42%). The increased antimicrobial resistance reflected in this study is in accordance with the findings of previous investigators.

Conclusion

Our study demonstrated that MALDI-TOF MS could identify a variety of non-fermenters from a wide range of infections. The most common isolate was Acinetobacter spp., followed by Pseudomonas spp. Many rare non-fermenters were also isolated. Majority of the strains were multidrug resistant to commonly used antibiotics such as cephalosporins, aminoglycosides and carbapenems. Our results show that identification of non-fermenters using MALDI-TOF MS is a reliable and rapid technique that shortens the time to initiate appropriate therapy and even the length of the hospital stay.

Conflict of Interest: None

Acknowledgment

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