

## Rapid Identification of Clinical Isolates of *Klebsiella pneumoniae* using MALDI-TOF MS from North India

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### Abstract:

Infections caused by multi-drug resistant (MDR) *Klebsiella pneumoniae* are increasing day by day. *K. pneumoniae* poses a severe public health concern and causes wide array of healthcare associated infections with limited treatment options. The quick detection of these isolates is of prime importance for the adoption of proper antibiotic treatment and control measures. Also the mis-identification using standard lab-based methods is quite common and hence, the clinical significance of *K. pneumoniae* complex members is inaccurately defined. Here, authors evaluated the potential of MALDI-TOF (Matrix-Assisted Laser Desorption Ionization-Time of Flight) MS (Mass Spectrometry) to discriminate and quick identification *K. pneumoniae* complex members. Thus, this study aimed at full MALDI-based approach to rapidly detect the *K. pneumoniae* isolates up to species level. We also explored the antibiotic resistance profile of *K. pneumoniae* isolates using CLSI (Clinical and Laboratory Standards Institute) guidelines. These results demonstrate the potential of MALDI-TOF MS for precise identification of *K. pneumoniae* complex members and could be effectively utilized in the routine diagnostic microbiology laboratories.

**Keywords:** *Klebsiella pneumoniae*, Multi-drug resistance, MALDI-TOF MS, Antibiotic susceptibility testing.

## INTRODUCTION

*Klebsiella pneumoniae* was first described by Edwin Klebs in 1875 from the respiratory tract of a pneumonia patient. *K. pneumoniae* species was formally described by Carl Friedlander in 1882 (Friedlaender, 1882). Escalated rates of multidrug-resistant (MDR) microorganisms are a global concern that necessitates novel way outs for the identification and investigation of antibiotic-resistant isolates (Jain and Mondal, 2007; Roca et al., 2015). *K. pneumoniae* is a challenging human bacterial pathogen, causing array of infections like; hospital-acquired infections (HAI) or community-acquired infections (CAI) that are associated with high rates of antibiotic resistance and human morbidity and mortality (Snitkin et al., 2012; Li et al., 2014; Wyres and Holt, 2016). The molecular revolution in clinical and diagnostic technology has been developed by many innovative technologies as Polymerase Chain Reactions (PCR), MALDI-TOF (Matrix-Assisted Laser Desorption Ionization-Time of Flight) MS (Mass Spectrometry), 16S rRNA and full genome sequencing, which help in identifying the pathogenic microorganisms. Sluggish phenotypic susceptibility testing methods and rapid but

selected resistance mechanisms targeting DNA/RNA based molecular assays are the main impediments for a rapid antibiotic treatment or initiation of infection control measures (Smiljanic et al., 2017). Early correct treatment is critical factor for a successful clinical outcome (Kumar et al., 2006). Recently, a worldwide spread of carbapenemase-producing *enterobacteriaceae* (CPE) occurred, primarily due to carbapenemase-producing *K. pneumoniae* isolates ( Vincent et al., 2009; Nordmann, Dortet, et al., 2012; Nordmann, Gniadkowski, et al., 2012; Nordmann, 2014; van Belkum et al., 2017).

The MALDI-TOF MS has transformed the routine microorganisms' identification, being a rapid and cheaper technique. Nowadays, it is characterized as a foremost identification method in many clinical microbiology laboratories ( Wenning et al., 2014; van Belkum et al., 2017). The MALDI-TOF MS worked on the principal of a proteotyping, high throughput analytical method based on the ionization and separation of ions according to their mass-to-charge ( $m/z$ ) ratio, originating a characteristic mass spectra of a given bacterial cell, which reflects mostly the content of peptides and small proteins (ribosomal proteins) (Singhal et al., 2015). MALDI-TOF MS is also being used to rapidly detect the antibiotic resistance by novel direct-on-target microdroplet growth assay (Idelevich et al., 2018). Recently, Youn et al. proposed that MALDI-TOF MS can be used to detect KPC production in *enterobacteriaceae* by revealing the presence of a specific single-peak marker (Youn et al., 2016). This study investigated that the MALDI-TOF MS method could be used in clinical microbiology laboratories for routine identification of *K. pneumoniae*. MALDI-TOF MS could be used in the rapid detection of this multidrug-resistant micro-organism both for epidemiological investigations and for the management of infections.

## MATERIALS AND METHODS

### Bacterial samples and growth conditions

A total of 20 samples of *K. pneumoniae* were collected consecutively from the Clinical Bacteriology Laboratory, Department of Medical Microbiology, MMDU, Mullana-Ambala, during February-April, 2019. Four types of clinical specimens were included in this study i.e. blood, pus, sputum, urine. Study was performed only on an already stored clinical sample; hence, patient-identifying information was not included. Blood specimens were cultured in the BD Bactec FX BC system (Becton Dickinson Instrument Systems, Sparks, MD, USA) and pus, sputum and urine specimens were cultured on routine blood agar and MacConkey agar plates. All positive cultures were subjected to Gram staining. On identification of Gram-negative rods; same were directly used for MALDI-TOF MS identification. Conventional cultures of the all other specimens were performed for the isolates' identification confirmation followed by biochemical methods and antimicrobial susceptibility testing. Bacterial isolates were grown on common microbiological media such as Blood Agar MacConkey Agar and Muller Hinton Agar (MHA) at 37°C for overnight.

### Preservation of isolates

All clinical isolates were grown on MHA (Muller Hinton Agar) plates and pure growths were stocked in Brain Heart Infusion (BHI) broth having 15% ( $v/v$ ) glycerol at -20°C freeze in duplicates.

### Identification of *K. pneumoniae* isolates

Various biochemical tests and gram staining procedures were performed on the clinical samples to differentiate them from other bacterial species and hence identify it. Bacterial colonies were further identified and characterized based on morphological and biochemical tests (Cheesbrough, 1989). These include Triple sugar iron (TSI) test, Citrate test, Urease test, Mannitol test and Indole test. All the results were further confirmed by MALDI-TOF-MS (Fiori et al., 2016).

### Antimicrobial susceptibility test

Aliquots from each positive culture were subjected to Gram staining and microscopy. All the isolates were sub-cultured in parallel on selective and nonselective routine agar plates and incubated under appropriate atmospheric conditions for 24hrs. Antimicrobial susceptibility of *K. pneumoniae* isolates to amoxicillin, cephalaxin, ceftriaxone, pip-taz, ampicillin, ciprofloxacin, cotrimoxazole imipenem, ceftazidime, cefotaxime, cefixime, chloropheniramine, meropenem, were determined by using the disc diffusion method on an in-house manufactured MHA ((Upadhyay, 2016a,b). It was carried out

according to the CLSI (Clinical and Laboratory Standards Institute) guidelines (CLSI, 2006). The results were interpreted according to the manufacturer's instructions. All chemicals required for culture media, reagents and antibiotic discs were procured from Hi-Media laboratories Pvt. Ltd., Mumbai.

**Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)**

MALDI-TOF MS analysis was performed using a Microflex LT mass spectrometer (Bruker Daltonics, Bremen, Germany) with a 60-Hz nitrogen laser to analyze spectra over a mass range of 2000-20000 Da. All specimens were processed as per the manufacturer's instructions. Pre-analytic preparation of samples was performed by using a sterile wooden tip to pick an isolated bacterial colony freshly grown on defined agar medium and then smearing a thin film in duplicate onto a ground steel MALDI biotarget 96 plate (Direct Transfer procedure). The microbial film was overlaid directly with 1.0µl α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution with the help of 1.0µl auto pipette. The sample-matrix mixture was dried at room temperature and subsequently inserted into the system for data acquisition. Spectra were analyzed with the Bruker's Biotyper software (version 3.1), and the Bruker Biotyper library V.4.0.0.1 (5627 entries) was used to achieve bacterial identification (Fiori et al., 2016). Manufacturer-recommended score cut-offs were used to determine genus level (1.7000 to 1.999) or species level (≥2.000) of the organism. A score of <1.7 was considered unreliable for genus identification.

**Table 1: Antibiotic susceptibility testing of *K. pneumoniae* isolates using disc diffusion assay.**

S. No.	Strain ID	Source	Panel of antibiotics used for susceptibility testing												
			a	b	c	d	e	f	g	h	i	j	k	l	m
1.	4002	Sputum	S	R	R	S	R	R	R	S	R	R	R	R	R
2.	4007	Blood	R	R	R	S	R	R	R	S	R	R	R	R	S
3.	4011	Sputum	R	R	R	S	R	R	R	S	R	R	R	R	S
4.	4449	Stool	R	R	R	S	R	R	R	S	R	R	R	R	S
5.	4437	Urine	R	R	R	R	R	R	R	S	R	R	R	R	S
6.	4421	Blood	R	R	R	S	R	R	R	S	R	R	R	R	S
7.	4350	Pus	R	R	R	R	R	R	R	S	R	R	R	R	S
8.	4351	Sputum	S	R	R	R	R	R	R	S	R	R	R	R	R
9.	4337	Urine	R	R	R	R	R	R	R	R	R	R	R	R	R
10.	4310	Pus	R	R	R	S	R	R	R	S	R	R	R	S	R
11.	4219	Blood	S	R	R	S	R	R	R	S	R	R	R	R	S
12.	4953	Pus	S	R	R	R	S	R	R	S	S	R	S	R	S
13.	4862	Urine	R	R	R	S	R	R	R	S	R	R	R	R	R
14.	4855	Blood	R	R	R	S	S	R	R	R	R	R	R	R	S
15.	4850	Urine	S	R	R	S	S	R	S	S	R	R	R	S	S
16.	4856	Stool	R	R	R	S	R	R	R	S	R	R	R	S	R
17.	4860	Urine	R	R	R	S	R	R	R	S	R	R	R	S	R
18.	4951	Blood	S	R	R	S	R	R	R	S	R	R	S	R	S
19.	4955	Blood	S	R	R	R	S	R	R	S	R	R	R	R	S
20.	4960	Urine	S	R	R	S	R	S	R	S	R	S	S	R	S

Where: a, Amoxicillin; b, Cephalaxin; c, Ceftriaxone; d, Pip-Taz; e, Ampicillin; f, Ciprofloxacin; g, Cotrimoxazole; h, Imipenem; i, Ceftazidime; j, Cefotaxime; k, Cefixime; l, Chloropheniramine; m, Meropenem; R, Resistant; S, Susceptible.

**RESULTS**

The AST (Antimicrobial Susceptibility Test) pattern of *K. pneumoniae* isolates in the present study revealed that all of isolates (100%) showed resistance to cephalaxin and ceftriaxone followed by 95% to ciprofloxacin, ceftazidime, cefotaxime and cotrimoxazole. The 85% isolates were resistant to

cefexime followed by ampicillin and chlorphenirmine (80%). Lowest to moderate resistance was observed against imipenem (10%) followed by Pip-Taz (30%) and meropenem (35%) (Table 1). All the 20 *K. pneumoniae* isolates were found to be MDR (multidrug resistant), which was defined as resistant to at least one agent in three or more antimicrobial classes. Majority of them were resistant to 9 or more antibiotics confirmed through the disc diffusion assay (Fig. 1). All the isolates were confirmed as Gram negative after preparing smear and performing Gram's staining followed by microscopy. The results of biochemical tests like Triple Sugar Iron (TSI) test, Citrate test, Urease test, Mannitol test, Indole test identified the strains as *K. pneumoniae*. The 20 strains of *K. pneumoniae* were correctly identified at the species level by MALDI-TOF MS, with score values above 2.0 (Table 2).

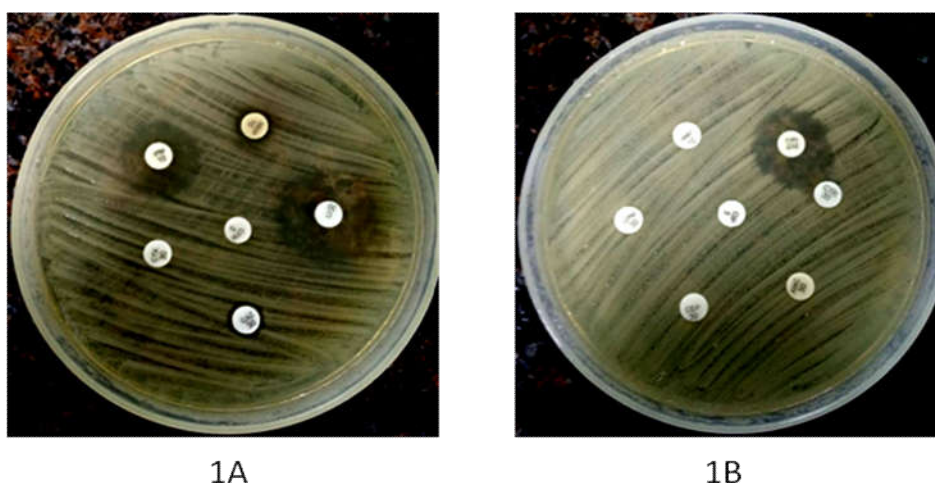


Figure 1: Disc diffusion assay showing resistant (no zone of inhibition) and susceptible (zone of inhibition) pattern of *K. pneumoniae* against different antibiotics.

Table 2: MALDI BioTyper identification score value of isolates and its best match organism.

S. No.	Strain ID	Organism (best match)	Score Value	S. No.	Strain ID	Organism (best match)	Score value
1.	4002	<i>Klebsiella pneumoniae</i>	2.052	11.	4219	<i>Klebsiella pneumoniae</i>	2.218
2.	4007	<i>Klebsiella pneumoniae</i>	2.092	12.	4953	<i>Klebsiella pneumoniae</i>	2.126
3.	4011	<i>Klebsiella pneumoniae</i>	2.288	13.	4862	<i>Klebsiella pneumoniae</i>	2.126
4.	4449	<i>Klebsiella pneumoniae</i>	2.028	14.	4855	<i>Klebsiella pneumoniae</i>	2.244
5.	4437	<i>Klebsiella pneumoniae</i>	2.094	15.	4850	<i>Klebsiella pneumoniae</i>	2.091
6.	4421	<i>Klebsiella pneumoniae</i>	2.062	16.	4856	<i>Klebsiella pneumoniae</i>	2.301
7.	4350	<i>Klebsiella pneumoniae</i>	2.094	17.	4860	<i>Klebsiella pneumoniae</i>	2.231
8.	4351	<i>Klebsiella pneumoniae</i>	2.121	18.	4951	<i>Klebsiella pneumoniae</i>	2.122
9.	4337	<i>Klebsiella pneumoniae</i>	2.133	19.	4955	<i>Klebsiella pneumoniae</i>	2.112
10.	4310	<i>Klebsiella pneumoniae</i>	2.221	20.	4960	<i>Klebsiella pneumoniae</i>	2.051

## DISCUSSION

The upsurge of MDR Gram-negative bacteria has a crucial role on clinical outcome, which portrays a dire healthcare problem. This study was aimed to investigate the antimicrobial resistance pattern and rapid identification of human clinical isolates of *K. pneumonia* collected from clinical bacteriology Lab, Department of Medical Microbiology, MMIMSR, Mullana, India. As we know, EUCAST faced inconsistent susceptibility results for colistin, BMD (Broth Micro-Dilution) characterized as the reference method for colistin AST. Newer and cheaper diagnostic methods could possibly overcome the rampant use of antibiotics, which further control the spread of antimicrobial resistance. Recent study showed MALDI-TOF MS fingerprinting as reliable techniques for identifying microorganisms to genus and species level, as well as for phylogenetic evolutionary studies (Barnini et al., 2015).

Analysis of sample was very quick: just about 90 min to prepare the samples on plates and examine a total 96 target; which was very crucial in reducing time for testing in a diagnostic laboratory. Moreover, very low cost of reagents underpins a very strong advantage (El-Bouri et al., 2012), over the traditional methods (Angeletti, 2017).

Antibiotic resistance is a major clinical problem in treating infection caused by these micro-organisms. The resistance to the antimicrobials has increased over the years. The recent emergence of a number of difficult-to-treat *K. pneumoniae* strains and infections is challenging the medical community to manage both host and bacterial factors critical during infection (Jain and Mondal, 2007; Roca et al., 2015). This study also reported the MDR isolates which are resistant to most of the antimicrobials. Continued studies on multi drug resistance *K. pneumoniae* are being done to investigate the resistance mechanism to identify the pathogen in patients. The MALDI-TOF-MS, a clinical set up used for the identification of bacteria has not only reduced the average turn-around time but also the cost per isolate with no loss in accuracy. MALDI-TOF- MS is a promising tool, but it needs optimization. There is a need to study problems and molecular epidemiology of *K. pneumoniae* to understand its resistance mechanisms and spread of different clones. Novel clones could also be noticed by performing MLST of PFGE techniques to study their spread.

## CONCLUSIONS

All the isolates were correctly identified as *K. pneumoniae* using MALDI-TOF MS, which were in the agreement of biochemical tests. Most of the isolates showed a high scale of resistance. To examine antibiotic profile, more number of isolates should be used in a large scale study. Regular check on antimicrobial susceptibility pattern and their judicious use is emphasized to minimize emergence of drug resistance *K. pneumoniae*. Molecular epidemiological studies could reveal the different resistant clones circulating in the existing hospital settings. Further, the RTSP MALDI-TOF detection assay could be used to predict KPC production with high accuracy in *K. pneumoniae*.

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