MALDI-TOF MS and Molecular methods for identifying Multidrug resistant clinical isolates of *Acinetobacter baumannii*

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Abstract

Acinetobacter baumannii has recently been reported as clinically most significant pathogen in the infections of hospitalized patients. It has become resistant to almost all classes of antimicrobials, which poses a threat in treating the patients with limited available therapeutic options. Rapid and accurate identification of this bug is foremost priority in treating patients infected with Acinetobacter species. Currently available phenotypic methods are unable to accurately identify and differentiate the closely related species of Acinetobacter. Advantages of molecular methods over conventional phenotypic methods were observed for accurate identification and segregation of various species of genus Acinetobacter.

Previous studies on Acinetobacter suggested that the discriminatory power of molecular methods like OXA-51 PCR and gyrb *multiplex PCR are higher than the phenotypic and conventional identification methods. MALDI-TOF MS has recently gained the attention of clinical fraternity towards quick, accurate and cheapest method for microbial identification, which also showed harmony with that of molecular methods. In the present study, we used multiple methods like phenotypic, biochemicals, MALDI-TOF MS,* bla$_{OXA-51}$*-like PCR and gyrB PCR methods to correctly identify A. baumannii and to analyze the precision of results obtained.*

Keywords: *Acinetobacter baumannii*, phenotypic methods, MALDI-TOF, molecular methods.

Introduction

Acinetobacter baumannii is a notorious pathogen which causes an array of infections in the hospitalized patients globally. VAP (Ventilator-associated pneumonia) and (BSI) blood stream infections are the most prevalent infections among others caused by *A. baumannii.*[1] *A. baumannii* is an aerobic, non-motile, catalase-positive, non-fastidious, glucose non-fermenter bacterium.[2] Rate of all the *A. baumannii* infections in hospital settings of Asian and Middle East is approximately 4% which is almost half in the Europe and United States.[3,4] At present, more than 50 *Acinetobacter* species have been classified and well designated. Majority of these are not pathogenic but few are selectively human pathogens. All the pathogenic species of *Acinetobacter* are classified as *Acinetobacter calcoaceticus baumannii* (Acb) complex.[5] Five major pathogenic species included in Acb complex are genomic species 3 (*A. pittii*), *A. baumannii*, genomic species 13 TU (*A. nosocomialis*), *A. seifertii* and *A. dijkshoorniae*.[6-8] *A. baumannii* affects majority of immune-compromised patients with weaker immune system. An array of resistance mechanisms and virulence factors enables *A. baumannii* to thrive in various healthcare settings. In the year 2017, WHO included *A. baumannii* in the top priority list of critical group of human pathogenic bacteria to direct the discovery of newer antimicrobial agents.[9]

Correct identification of *Acinetobacter* species is of prime importance to coorelate antimicrobial susceptibility with different genomic species.[10, 11] Due to the existence of many closely associated species, it is hard to differentiate the taxonomy *of Acinetobacter* by phenotypic and biochemical procedures. Nowadays, several molecular methods have been developed, which are used to identify the bacterium up to species level but most of them are time-consuming, laborious and cannot be implemented in routine laboratory. These include MLST (multi-locus sequence typing), RAPD (random amplified polymorphic DNA), PFGE (pulsed-field gel electrophoresis), AFLP (amplified fragment length polymorphism analysis), ribotyping, MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight) mass spectrometry, rep-PCR (repetitive extragenic palindromic sequence-based PCR), RNA Spacer fingerprinting, *rpoB* gene sequencing and 16S-23S rRNA sequence analysis.[12-18]

Moreover, the incidence of community-acquired *A. baumannii* infections has been gradually increasing.[2] A number of virulence factors like outer membrane porins, proteases, phospholipases, capsular polysaccharides, LPS (lipopolysaccharides), PSS (protein secretion systems) and siderophores (iron-chelating systems) have been detected by phenotypic and genomic analyses.[2,19]

A. baumannii is declared by WHO as the most critically significant ESKAPE pathogens, which efficiently abolishes the effects of an array of antimicrobials.[20] An array of antimicrobial resistance mechanisms have been traced in *A. baumannii* which includes outer membrane permeability defects, over-expression of efflux pumps, enzymatic degradation of drugs by beta-lactamases and target

modifications, multidrug.[2,21] In this study, we compared the precision of *Acinetobacter* spp. identification using MALDI-TOF MS, phenotypic, biochemical and molecular methods. Quick and correct identification of *Acinetobacter* spp. would be critically beneficial in treating deadly infections caused by the latter.

Material and Methods

Bacterial Strains and Sample Size: A total of 18 *A. baumannii* isolates were collected consecutively from the clinical bacteriology laboratory, Department of Medical Microbiology, MMIMSR, Mullana. Four types of clinical samples were received in this study i.e. blood, sputum, urine and ear swab. Bacterial isolates were grown on common laboratory media such as MacConkey agar, blood agar and MHA (Muller Hinton Agar) at 37°C for overnight.

Identification of bacterial isolates: Various biochemical tests and gram staining procedures were performed on the clinical samples to identify and differentiate them from other bacterial species and hence to identify them. These include TSI (triple sugar iron), citrate, urease, mannitol and indole tests. Further, MALDI-TOF MS followed by *OXA-51* and *gyrB* gene PCRs were also performed for confirming the identification of *A. baumannii* isolates.

MALDI-TOF MS: The Microflex LT MALDI-TOF MS (Bruker Daltonics, Germany) with a 60-Hz nitrogen laser was used to analyze spectra over a mass range of 2000-20000 Da. All specimens were processed as per manufacturer's instructions. Sample preparation was performed with the help of a sterile wooden tip to pick an isolated freshly grown bacterial colony. A uniform smear was prepared in the form of a thin film onto a MALDI steel plate (Direct Transfer procedure). 1.0 µl of α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution was tranfered onto the microbial films directly with the help of an autopipette.

The prepared mixture of sample-matrix was air dried at room temperature followed by insertion of the plate into the MALDI-TOF system for analysis. Resulted spectra were analyzed using MALDI Biotyper database version 3 (Bruker Daltonics, Germany). Genus level (1.7000 to 1.999) and species level (≥2.000) cut-offs scores were used to identify the *Acinetobacter* species as per manufacturer protocol. A score of <1.7 was considered unreliable for genus identification.

Molecular methods of identification: Several molecular methods like 16S rRNA, *recA* and 16S–23S ITS are available to identify any bacterial species upto species level. Herein, *OXA-51* gene and *gyrB* gene multiplex PCR were used to molecularly identify and confirm the species of *Acinetobacter* genus using primers given in the table 1.[22,23] DNA was isolated using heat-shock methods followed by DNA quantification using Nanodrop spectrophotometer. PCR master mix was prepared in 25 µl reaction volume containing 250 µM of each dNTP, 20 mM Tris–HCl, 0.5 µM of each primer, 2 mM $MgCl_2$, 50 mM KCl, nuclease free water and 1.0 U of Taq DNA polymerase (Sigma Alrich, St. Louis, Missouri, United States) and 1 µg of template DNA.

PCR amplification was performed at initial denaturation at 95 °C for 5 minutes followed by 35 cylces of 95 °C for 30 seconds, annealing at 62 °C for 30 seconds (*gyrb*), extension at 72°C for 30 sec and final extension at 72°C for 10 min. Same PCR profile was used for OXA-51 except the annealing temperature of 57 °C for 30 sec. PCR amplified products were analysed on 1.2% agarose gel electrophoresis. Gels were viewed under BioRad trans-illuminator and images were stored.

Preservation of Isolates: All clinical isolates were grown on MHA plates and pure growths were stocked in the BHI (Brain Heart Infusion) broth having 15% v/v glycerol at -20 °C freeze in duplicates.

Antimicrobial Susceptibility Test: *A. baumannii* antimicrobial susceptibility was determined using Kirby Bauer disc diffusion method for Amoxicillin, Cephalothin, Ceftriaxone, PIP-TAZ, Ampicillin, Ciprofloxacin, Cefepime, Gentamycin, Tetracyclin, Tobramyclin, Imipenem, Ceftazidime, Cefotaxime, Cefixime , Chloramphinicol, Meropenem antimicrobials on Mueller-Hinton agar. The results were interpreted according to the manufacturer's instructions and CLSI guidelines (Table 3).

Results

All the isolates were initially identified as gram negative based on staining and microscopy followed by biochemical testing like TSI, citrate, urease, mannitol and indole tests. Results of the biochemical tests are illustrated in table 2.

Table 1
Primer sequences and products of *OXA-51* and *gyrB* multiplex PCRs.

Genes	Sequence (5'→3')	Product
OXA-51_F	TAATGCTTTGATCGGCCTTG	353 bp[22]
OXA-51_R	TGGATTGCACTTCATCTTGG	
sp4-F	CACGCCGTAAGAGTGCATTA	sp4F + sp4R = 294 bp
sp4-R	AACGGAGCTTGTCAGGGTTA	
sp2-F	GTTCCTGATCCGAAATTCTCG	sp2F + sp4F + sp4R = 294 + 490 bp (multiplex) specific for *A. baumannii*.[23]

16 isolates were identified as *A. baumannii* two as *A. pittii* using MALDI-TOF MS with cut off score values above 1.9 for all strains (Table 4). After that, *OXA-51* and *gyrB* gene PCR methods confirmed the results of MALDI-TOF MS (Figure 1-2). *GyrB* gene PCR showed miscorrelation with *OXA-51* PCR and MALDI-TOF for one isolate where a faint band was observed (Figure 1-2). However, 15 isolates were correctly identified as *A. baumannii* and two as *A. pittii* by *OXA-51* and *gyrB* gene PCRs.

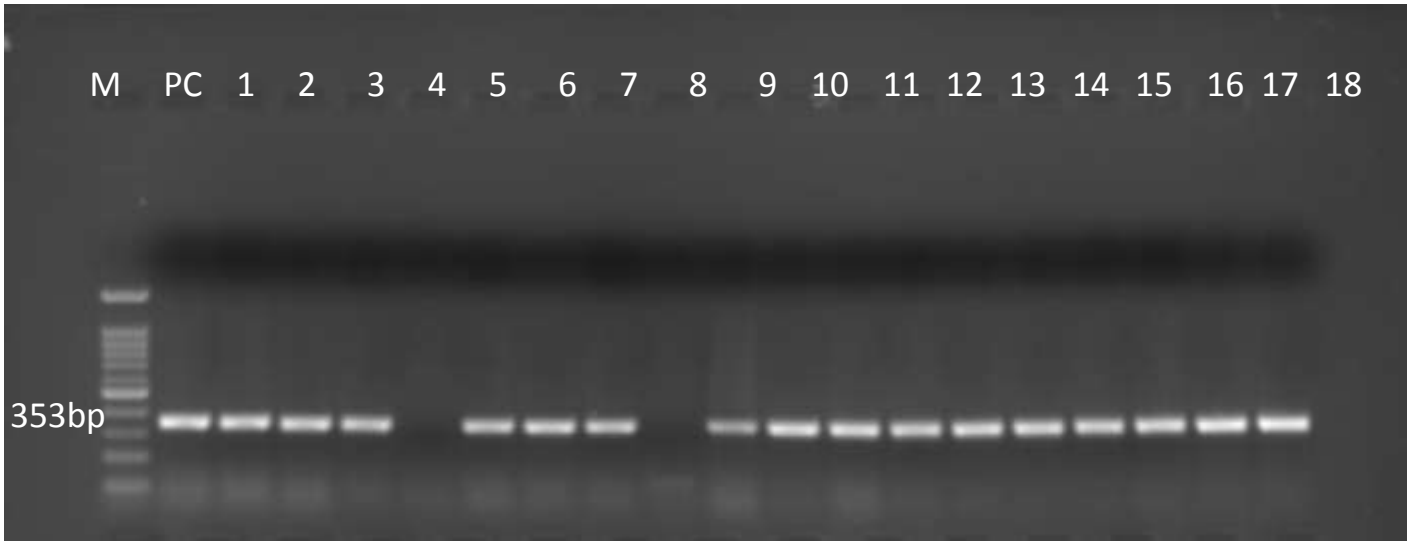

Figure 1: Gel image of *A. baumannii* Identification with *OXA-51* PCR. M – 100b-p ladder, PC positive control, Lanes 4 and Lane 8 non-*baumannii* species.

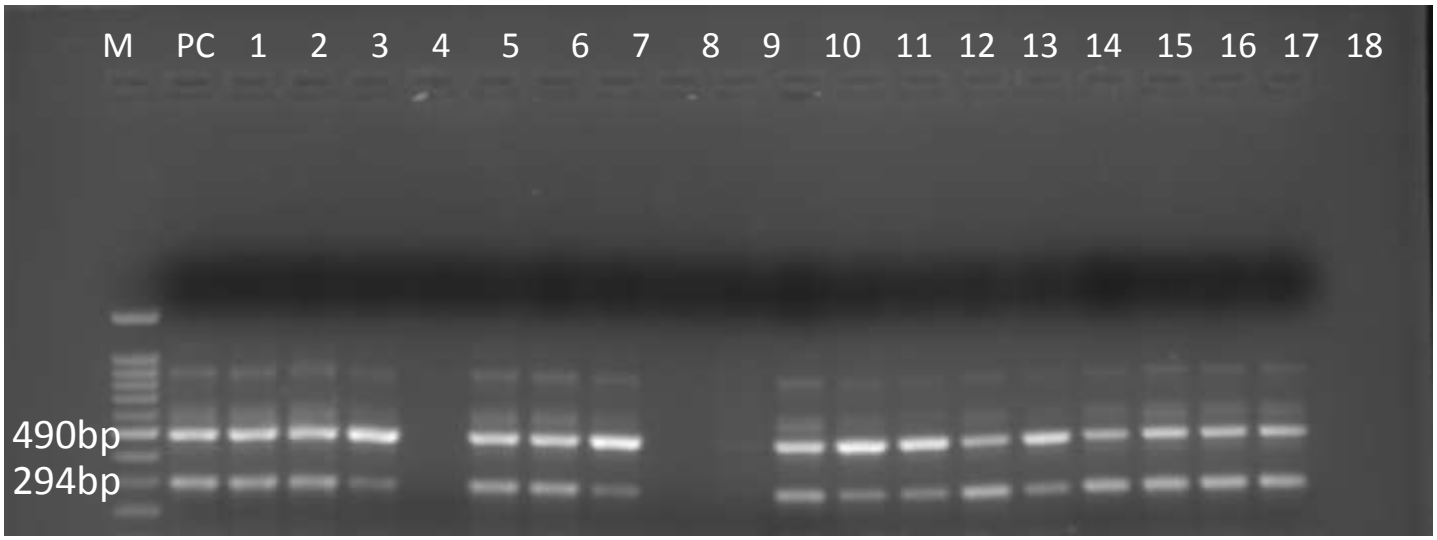

Figure 2: Gel image of *A. baumannii* Identification with *GyrB* PCR. M – 100b-p ladder, PC positive control. Lanes 4 and Lane 8 non-*baumannii* species, Lane 9 faint band showed false result.

Table 2
Biochemical tests and interpretations for *A. baumannii*.

Biochemical Tests	**Results**	**Interpretation**
Triple Sugar Iron [24]	K/K (Red to Red)	No Fermentation; Peptone Catabolized
MRVP	Negative	-
Indole	Negative	No indole production
H_2S	Negative	-
Motility	Negative	Non-motile
Oxidase	Negative	-
Catalse	Positive	-
Citrate	Positive (Green to Blue)	Utilize citrate as a carbon source
Urease	Negative (No colour change)	No ammonia production

MALDI-TOF MS and *OXA-51* PCR were found to fetch the accurate results (Table 4, Figure 1). It was also observed that all the isolates of *A. baumannii* and *A. pittii* were multidrug resistant by antimicrobial susceptibility testing (Table 3).

Discussion

Among all the species, *A. baumannii* is considered as the most significant species in the healthcare settings. Escalated resistance to various classes of antimicrobials has suggested the introduction of novel strategies to cure infections by this notorious pathogen. *A. baumannii* has got unique properties due to which different clones of it are found circulating in hospitals all over the globe. International clones I, II and II are the most prevalent ones which have been traced in the epidemic spread of *A. baumannii*.[25] High mortality and morbidity rates due to *A. baumannii* infections raised the dire need of prompt identification of this bacterium.

Few molecular studies like DNA-DNA hybridization, PCR sequencing of *rpoB* genes are accurate but demand much labour and high cost, which cannot be afforded in routine microbiology laboratories.[12-18] *OXA-51* and *gyrB* multiplex PCRs are also reliable molecular methods for identification but involved high cost and time.[22,23]

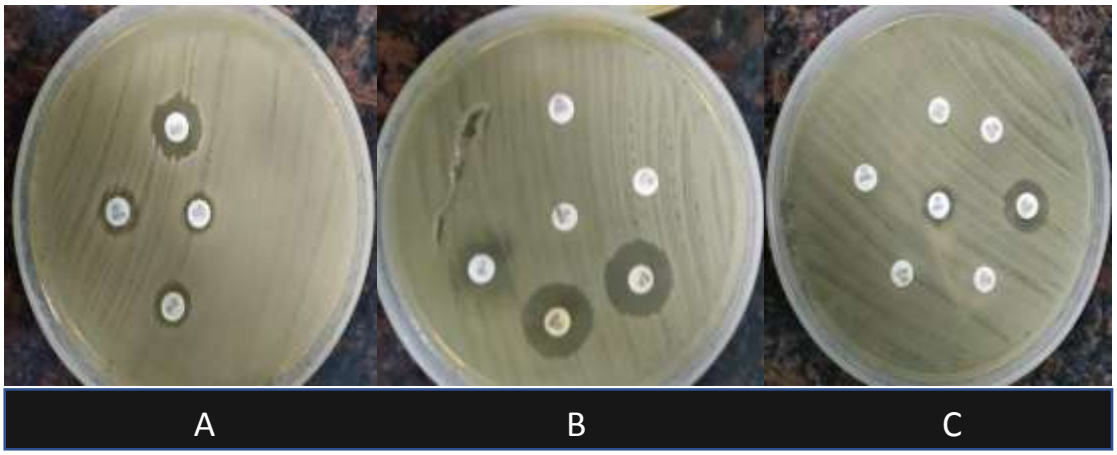

Figure 3: Cultured plates showing results of Kirby Bauer Disk Diffusion Assay.

Table 3
Results of Kirby Bauer Disk Assay.

Isolate No.	Source	Amoxicillin	Cephalothin	Ceftriaxone	PIP-TAZ	Ampicillin	Ciprofloxacin	Cefepime	Gentamycin	Tetracyclin	Tobramyclin	Imipenem	Ceftazidime	Cefotaxime	Cefixime	Chloramphinicol	Meropenem
1	Urine	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R
2	Blood	R	R	R	R	R	R	R	S	R	R	S	R	R	R	R	S
3	Blood	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	S
5	Sputum	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	S
6	Sputum	R	R	R	R	R	R	R	S	R	R	S	R	R	R	R	S
7	Sputum	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	S
9	Ear Swab	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	S
10	Sputum	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R
11	Urine	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R
12	Blood	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	S
13	Sputum	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	S
14	Sputum	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R
15	Urine	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R
16	Blood	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	S
17	Blood	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
18	Urine	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

Table 4
MALDI-TOF MS results of *Acinetobacter* species.

Analyte ID	Organism (best match)	Score Value
1	*Acinetobacter baumannii*	2.082
2	*Acinetobacter baumannii*	2.250
3	*Acinetobacter baumannii*	2.305
4	*Acinetobacter pittii*	2.332
5	*Acinetobacter baumannii*	1.917
6	*Acinetobacter baumannii*	2.305
7	*Acinetobacter baumannii*	2.061
8	*Acinetobacter pittii*	1.989
9	*Acinetobacter baumannii*	2.285
10	*Acinetobacter baumannii*	2.073
11	*Acinetobacter baumannii*	2.234
12	*Acinetobacter baumannii*	2.305
13	*Acinetobacter baumannii*	1.932
14	*Acinetobacter baumannii*	1.977
15	*Acinetobacter baumannii*	2.305
16	*Acinetobacter baumannii*	2.131
17	*Acinetobacter baumannii*	1.971
18	*Acinetobacter baumannii*	2.162

OXA-51 gene, which was once considered to be specific to *A. baumannii*, now has been reported in the *A. nosocomialis* and other related speacies.[26, 27] Mobile genetic element ISAba1 located upstream to *OXA-51* is responsible for the horizontal transfer of the latter from one to another species with the help of transposon *Tn6080*.[28]

This study was designed to correctly and quickly identify the clinical isolates of *Acinetobacter* species using different methods. We also observed the comparative time and the cost involved in the different methods. We investigated 18 isolates of *Acinetobacter* species received from different clinical specimens. MALDI-TOF MS was considered as the most cost effective method with least time required in identifying the species of genus *Acinetobacter*. Results were confirmed with the molecular methods using *OXA-51* and *gyrB* gene PCRs. *OXA-51* PCR showed high (100%) sensitivity for identification of *A. baumannii* and *A. pitti* isolates. However, a large scale study with large number of isolates would be vital to cross verify the findings of present study.

Conclusion

In the present study, we have identified and validated the *Acinetobacter* species identification by multiple methods. MALDI-TOF MS reproduced the accurate results which were obtained through *OXA-51* gene PCR. However, *gyrB* gene PCR also reproduced the same result except one isolate which was reported as non-*baumannii* species of *Acinetobacter*. It was also observed that all the clinical isolates of *A. baumannii* were multidrug resistant. Since, MALDI-TOF MS has been observed as a good candidate for excellent species identification results; it can further be used for quick and reliable identification of *A. baumannii* in the routine microbiology laboratory. This can be of immense significance to reduce the time in prescribing antimicrobial therapy to critically ill patients of *A. baumannii* infections the identification of this species.

This study would be of critical significance in the clinical settings where prompt identification is required for immediate and accurate therapeutic interventions. MALDI-TOF MS could further be useful in illustrating epidemiology of this species in the different sites of hospital.

Acknowledgement

We thank Dr Varsha Sigh, HOD, Department of Microbiology, MMIMSR, Mullana (Ambala) for providing us the strains. We are extremely thankful to Professor Pallab Ray for helping us in the identification of isolates at Department of Medical Microbiology, PGIMER, Chandigarh using MALDI-TOF MS facility.

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(Received 26th July 2020, accepted 27th August 2020)