

Epidemiological Molecular Study of Metallo Beta Lactamases in *Acinetobacter Baumannii* at a Tertiary Care Hospital

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Abstract:

Background and Objectives: Carbapenem resistance mediated by metallo beta lactamases (MBL) in *Acinetobacter baumannii* is a global challenge due to its rapid spread and limited therapeutic options. To determine the prevalence of MBL in *A. baumannii* isolates in hospitalized patients by both phenotypic and genotypic methods.

Materials and Methods: The clinical samples were collected from in patients and subcultured on routine culture media for growth. Identification of bacteria along with antimicrobial sensitivity testing was done by VITEK -2 Compact (bioMerieux). Antibiotics that were not tested by VITEK-2 were tested manually by Kirby-Bauer disk diffusion method according to CLSI 2017 and EUCAST 2016 guidelines. The isolates which were resistant to carbapenem (imipenem and/ or meropenem) were tested by phenotypic (imipenem-EDTA combined disk method) and genotypic method for presence of common metallo beta lactamases genes (*blaIMP*, *blaNDM*, *blaGIM*, *blaVIM*, *blaSPM* and *blaSIM*).

Conclusion: The prevalence of MBL in carbapenem resistant isolates of *A. baumannii* was 87.7%. *blaNDM* was the most common gene detected. No significant difference was found in the ability of phenotypic and genotypic methods for MBL detection. The resistance rate of the *A. baumannii* is high for most antibiotics except for polymyxins (E&B) and tigecycline.

Keywords: Metallo beta Lactamases, *Acinetobacter baumannii*, Carbapenem..

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Introduction

Acinetobacter baumannii is a nosocomial pathogen that is widely reported from hospitals, especially in ICU. The reports of multidrug-resistant isolates have increased during the last decade, probably due to increasing use of antimicrobial agents. [2]

Carbapenems are used as the last resort antibiotics for treatment of these infections, however in the recent years many reports show increasing resistance to them as well [2]. Resistance to carbapenems in *A. baumannii* may be due to various reasons like over-expression of an efflux pump, loss or reduced expression of the porin proteins and production of metallo beta lactamases that hydrolyse carbapenems (carbapenemases). [3] Metallo beta lactamases (MBL), are Ambler class B enzyme and include imipenemase (IMP), Verona imipenemase (VIM), Seoul imipenemase (SIM) and New Delhi metallo beta-lactamase (NDM)-1. They have been identified in *A. baumannii* throughout the World [4, 5]. The MBL encoding genes are located on integrons that are capable of horizontal gene transfer [5]

This makes it important to detect MBL in *A.*

baumannii, which could help in control and prevent further dissemination of such mobile genes [6]. Various studies have been conducted across India with a prevalence of 9.3%-70.9% in different states [6-8]. The common genotypes reported are *blaIMP* and *blaVIM* [7]. In Gujarat, the reported prevalence of MBL in *A. baumannii* is 29.6% [9] but the genotypes responsible for MBL resistance in Bihar state of India, is still unexplored. This study was undertaken to detect the MBL encoding genes (*blaIMP*, *blaNDM*, *blaGIM*, *blaVIM*, *blaSPM* and *blaSIM*) in *A. baumannii* isolates in a tertiary care hospital located in rural part of Bihar.

Materials and Methods

A cross-sectional study was conducted. Department of Microbiology, at Darbhanga medical college and Hospital Laheriasarai Darbhanga, Bihar. Study duration is Two years. The clinical samples like pus, endotracheal aspirates, blood, sputum, body fluid etc. were collected from inpatients and subcultured on routine culture media for growth. Identification of bacteria along with antimicrobial sensitivity

testing was done by VITEK -2 Compact (bioMerieux). Antibiotics that were not tested by VITEK-2 were tested manually by Kirby-Bauer disk diffusion method according to CLSI 2017 and EUCAST 2016 guidelines (only for tigecycline) [10, 11.] The antibiotics that were tested by VITEK-2 Compact by using GN280 card included cefepime, ceftriaxone, ciprofloxacin, colistin, gentamicin, imipenem, levofloxacin, meropenem, piperacillin-tazobactam, tigecycline, trimethoprim-sulfamethoxazole, while the rest of antibiotics such as amikacin (30µg), doripenem (10µg), ampicillin-sulbactam (10/10µg), doxycycline (10µg), minocycline (30µg), netilmicin (30µg), piperacillin (100µg), polymyxin B (MIC) and tobramycin (10µg) were procured commercially from Hi-media laboratories limited, India and tested by disk diffusion method. The isolates which were resistant to carbapenem (imipenem and/ or meropenem) were tested by phenotypic (imipenem-EDTA combined disk test) method and genotypic by uniplex PCR to detect MBL genes such as *blaGIM* and *blaNDM* and multiplex PCR to detect other MBL genes such as, *blaIMP*, *blaVIM*, *blaSIM*, and *blaSPM*.

Imipenem-EDTA combined disk test (IMP-EDTA CDT) [12]:

The suspension was prepared from an overnight bacterial culture and inoculated on Mueller-Hinton

agar. 10µg imipenem disc and an imipenem-EDTA (10/750µg) disc were placed on it. After overnight incubation at 35°C, the established zone diameter difference of ≥ 7 mm between imipenem disc and imipenem plus EDTA disc was interpreted as MBL positive.

PCR amplification protocols: DNA extraction was done by boiling lysis method. The cell suspension from an overnight culture was boiled in 500 µl saline at 100°C for 10 minutes, then it was placed at room temperature for five minutes. The suspension was centrifuged at 8,000 rpm for 5 minutes. The supernatant was used as a template for PCR amplification [13]. The PCR reaction mixture consisted of 10 µl of PCR master mix (2x), 3 µl distilled water,

1.0 µl of each primer (forward and reverse) and 5 µl of DNA in a total volume of 20 µl.

The PCR thermal cycling conditions:

For *blaVIM*, *blaSPM*, *blaSIM* and *blaIMP* genes, thermal profile was set according to the previously reported work by Mohanam *et al* [14]. Thermal conditions for two genes, *blaNDM* and *blaGIM*, were standardised using gradient PCR for their annealing temperature, the final conditions were set as shown in the table 1.

Table 1: Steps and conditions of thermal cycling for *blaNDM* and *blaGIM* primer genes in PCR.

Genes	Steps	Cycling conditions		
		Temperature	Time	
<i>blaNDM</i>	Initial Denaturation	95 °C	5 min	Repeated for 35 cycles
	Denaturation	95°C	0:15 sec	
	Annealing	55°C	0:20 sec	
	Extension	72°C	0:40 sec	
	Final Extension	72°C	5 min	
<i>blaGIM</i>	Initial Denaturation	95°C	5 min	Repeated for 35 cycles
	Denaturation	95°C	0:15 sec	
	Annealing	57°C	0:30 sec	
	Extension	72°C	0:45 sec	
	Final Extension	72°C	5 min	

The PCR was considered as the gold standard for MBL detection. On the basis of the phenotypic and/ or genotypic results of *A. baumannii* isolates were divided into two groups i.e. MBL-positive and MBL-negative strains. Statistical analysis was performed using SPSS software version 20.0. The

results were presented as descriptive statistics in terms of relative frequency. Chi-square test was used to analyse the results wherever they needed. P value of < 0.05 was considered as statistically significant.

Table 2: Details of MBL genes specific primers used.

Name of primers	Primer sequence (5'-3')	Product length	Reference
<i>blaNDM</i> F	GGGCAGTCGCTTCCAACGGT	476 bp	Shenoy <i>et al.</i> [15]
<i>blaNDM</i> R	GTAGTGCTCAGTGTCCGGCAT		
<i>blaGIM</i> F	AGAACCTTGACCGAACGCAG	749 bp	Castanheira <i>et al.</i> [16]
<i>blaGIM</i> R	ACTCATGACTCCTCACGAGG		
<i>blaVIM</i> F	GATGGTGTITGGTCCGATA	390 bp	
<i>blaVIM</i> R	CGAATGCGCAGCACCAG		

<i>bla</i> IMP F	GGAATAGAGTGGCTTAAYTCTC	232 bp	Mohanam <i>et al.</i> [14]
<i>bla</i> IMP R	GGTTTAAAYAAAAACAACCACC		
<i>bla</i> SIM F	TAC AAGGGATTTCGGCATCG	570 bp	
<i>bla</i> SIM R	TAATGGCCTGTTCCCATGTG		
<i>bla</i> SPM F	AAAATCTGGGTACGCAAACG	271 bp	

Results

During the study period 2843 non duplicate clinical specimens were processed for culture and sensitivity test that yielded 947 (33.3%) pathogenic organisms. 737(77.8%) out of 947 were found to be gram negative bacteria, of which 84 (11.4%) were *A. baumannii*. Table 3, illustrate the sample-wise distribution of MBL-positive and MBL-negative *A. baumannii* isolates. The majority i.e. 40(47.6%) of samples source were tracheostomy/endotracheal/broncho alveolar lavage (TT/ET/BAL) followed by sputum, blood and pus/swab i.e. 18(21.4%), 12(14.3%) and 12(14.3%) respectively. For MBL positive isolates, also the majority i.e. 34 (85.0%) of samples source were (TT/ET/BAL) followed by blood and sputum i.e. 10 (83.3%, each). Table 4, illustrate the

distribution of carbapenem resistant isolates between phenotypic and genotypic detection methods. Out of 73 carbapenem resistant isolates only 28 isolates were positive by both phenotypic and genotypic method leading to a sensitivity and specificity of 87.5% & 21.95% by IMP- EDTA CDT, when compared to genotypic method as gold standard. The genes that were detected among IMP-EDTA CDT positive and negative isolates of *A. baumannii* included *bla*NDM in 29(39.7%), *bla*GIM in 2(2.7%) and *bla*VIM in one isolate (1.4%) (Table 5). No isolates were found positive for *bla*SIM, *bla*SPM and *bla*IMP genes. Four isolates with positive genes (three *bla*NDM & one *bla*VIM) were negative by phenotypic method.

Table 3: Distribution of clinical samples among MBL positive & MBL negative isolates of *A. baumannii* (n=84).

Specimen	MBL Positive n=64 (%)	MBL Negative n=20 (%)	Total n=84 (%)
Sputum	10 (55.6)	8 (44.4)	18 (100)
TT/ET/BAL	34 (85.0)	6 (15.0)	40 (100)
Pus/Swab	9(75.0)	3 (25.0)	12 (100)
Blood	10 (83.3)	2(16.7)	12 (100)
Body fluids	1 (50.0)	1 (50.0)	2 (100)

TT/ET/BAL: Tracheostomy/endotracheal/bronchoalveolar lavage

Table 4: Comparison between phenotypic and genotypic MBL detection method among carbapenem resistant isolates of *A. baumannii* (n=73).

Phenotypic test	PCR+ve	PCR-ve	P value
IMP-EDTA +ve=60(%)	28(46.7)	32 (53.3)	0.295
IMP-EDTA-ve=13(%)	4(30.8)	9(69.2)	
Total= 73 (%)	32 (43.8)	41(56.2)	

Sensitivity=87.5%, specificity =21.95%, negative predictive value=69.23%, positive predictive value=46.67%.

Table 5: Expression of MBL genes among IMP-EDTA CDT positive and negative isolates of *A. baumannii*.

MBL genes	Detected by IMP-EDTA CDT	Not detected by IMP-EDTA CDT	Total=32
<i>bla</i> NDM=29(%)	26(89.7)	3(10.3)	29(100)
<i>bla</i> GIM=2(%)	2(100)	0(0.0)	2(100)
<i>bla</i> VIM=1(%)	0(0.0)	1(100)	1(100)
Total=32(%)	28(87.5)	4(12.5)	32(100)

Discussion

Infection by multidrug resistant (MRD) *A. baumannii* along with metallo beta lactamases activities represent the therapeutic trouble in hospital environment especially in ICU [5].

A. baumannii has been isolated from almost all samples as it has the potential to cause wide variety of clinical manifestations. Respiratory tract infections have been reported to be the commonest of

these[5,17]. In the present study, this is supported as 69% isolates of *A. baumannii* were obtained from the respiratory specimen. This is also consistent with data presented by other investigators where more than 60% isolates were from respiratory specimens though blood has also been reported as the major clinical source of *A. baumannii* [5,17,18]. An alarming rise in antibiotic resistance rate of *A. baumannii* has now become a serious and an increasingly common public health concern [18]. In the current study,

antibiotic resistance rate in *A.baumannii* is high ranging from 73.8-98.8% for most antibiotics except for colistin, polymyxin B and tigecycline. This is similar to findings by Saranya A *et al*, where resistance to most of the antibiotics was in the range of 62.5 to 100% [19]. The antibiotics resistance is higher among the MBL positive group as compared to the MBL negative group, which is in agreement with findings by Singla P *et al* and by Nandy S *et al* [8,20]. Carbapenem resistance in *A.baumannii* has been reported in various studies, ranging from 40-98.7% [5] We found 86.9% carbapenem resistance in *A. baumannii* which is similar to 85% resistance reported by Rynga D *et al* (2015) from Delhi. But previous studies conducted in our institute by Pandya *et al*, (2012) and Kalpesh *et al* (2016) have reported a comparatively higher resistance to carbapenems i.e. 98.8% and 88.5% emphasizing the need to keep a watch on current rates.[9] In CLSI guidelines, no standard phenotypic method has been given for detection of MBL [8]. The most commonly used method is IMP-EDTA CDT and has variable sensitivity & specificity when compared with genotypic methods as gold standard. In a study conducted by Kumar H *et al*, the sensitivity and specificity of IMP-EDTA CDT was 86.2% and 30.9% respectively, which is similar to our finding of 87.5% and 21.95% respectively. A very high sensitivity and specificity of IMP-EDTA CDT ranging from 96.11-100% has also been reported in the studies conducted by Sachdeva *et al* and Franklin C *et al*). We considered any isolates as MBL positive for MBL production if positive by either or both phenotypic and genotypic methods. The rates of MBL positivity in *A.baumannii* is highly and ranges from 9.3-70.9% at different geographic locations in India. In the present work, 87.7 % isolates of carbapenem resistant *A.baumannii* were positive for MBL production, Similar higher rates have been reported from Iran (86.8%) and Egypt (97.5%).

The common gene reported from Asia include *blaIMP* and *blaVIM*. *BlaIMP* is found mainly in Japan, Korea, China, Taiwan, and Iran. The most recently described MBL, namely New Delhi Metallo beta-lactamase (NDM) has been reported from the Indian subcontinent, the Balkans regions, Southeast Asia and the Middle East. In our study, the most predominant gene was *blaNDM* (39.7%), similar to finding by Vijayakumar *et al* (2016) from Tamil Nadu and Solanki R *et al* (2014) from Hyderabad i.e. 19.2% and 36.4% respectively. Some of the studies in India e.g. Nandy S *et al* (2015) and Goudarzi H *et al* (2015) have not yet detected NDM gene at their locations. We detected *blaGIM* gene in 2.7%, which is considerable lower than 24.0% reported by Rynga Det al (2015) from Delhi. A study from Egypt by Alkasaby NM *et al* (2017) has reported a much higher rate of 42.9 % for *blaGIM* but is still not detected in a study conducted by Kock MM *et al* (2013) from South Africa. We had 1.4% isolates

positive for *bla VIM* gene which is similar to finding by Ryoo NH *et al* (2010) from Korea.

Conclusion

The prevalence of metallo beta lactamases is high (86.9%) in our setting and there is a difference in the ability of phenotypic (82.2%) and genotypic methods (43.8%) for MBL detection. *blaNDM* is the most common gene (39.9%) followed by *blaGIM* (2.7%) and *blaVIM* (1.4%). The clinical isolates of *A. baumannii* showed a high resistance rate to most of antibiotics except for polymyxins and tigecycline.

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