

Prevalence of Carbapenemases with Detection of NDM-1 Gene in Nonfermenters Isolated from a Tertiary Care Hospital of North India

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ABSTRACT

Background: Increasing multidrug resistance in nonfermenters is of great concern to mankind because the drugs left in pipeline are only a few for the serious infections in hospital ICU settings. The present study was performed to determine the prevalence of carbapenem resistance among nonfermenters and to characterize the most prevalent MBL genes (NDM-1) by polymerase chain reaction, among phenotypically MBL positive isolates in a tertiary care centre of north India.

Methods: A total of 383 clinical isolates comprising of 181 *A. baumannii* and 182 *P. aeruginosa* were included in this study. The *A. baumannii* and *P. aeruginosa* were identified by using standard bacteriological techniques. All these isolates were screened for imipenem and meropenem resistance by Kirby-Bauer disk diffusion method and Minimal Inhibitory Concentration (MIC) methods according to CLSI guidelines.

Results: In the present study, the two nonfermenters (*A. baumannii* and *P. aeruginosa*) showed highest susceptibility to polymyxin-B (78.2%) followed by meropenem (70.5%), imipenem (66.4%), tigecyclin (57.6%) and combination of piperacillin-tazobactam (51.5%) by using disc diffusion method.

Conclusion: Treatment of infections caused by nonfermenters like *P. aeruginosa* and *A. baumannii* producing different carbapenemases including NDM-1 are now posing serious challenge as these infections are resistant to almost all available antimicrobial agents.

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Introduction

Non Fermentative Gram-Negative Bacilli (NFGNB) are generally saprophytic in nature but can cause a significant number of infections, particularly in the immunocompromised patients. *Pseudomonas aeruginosa* and *Acinetobacter baumannii* are the commonest nonfermenters implicated in such infections. Infections caused by other species are relatively infrequent.^[1] Resistance to carbapenems is of great concern as these are considered to be the antibiotics of last resort to combat infections by multidrug resistant bacteria in recent days, especially in ICUs and other high risk areas of a hospital.^[2] Resistance to carbapenem occurs due to decreased outer membrane permeability, increased efflux system and emergence of carbapenem hydrolyzing enzymes.^[3] Out of huge spectrum of such prevalent carbapenemases, metallo beta lactamases (MBL) are one of the most important enzyme.

Therefore it seems imperative to see presence of resistance against different antibiotics specially imipenem and meropenem in the two non-fermenters that are notoriously known for very high resistance. Keeping in mind the above factors, this study was planned to see overall prevalence of carbapenem resistance with special reference to MBL including bla_{NDM} in the tertiary level hospital of North India.

Materials And Methods

A total of 383 clinical isolates comprising of 181 *A. baumannii* and 182 *P. aeruginosa* were included in this study. These organisms were isolated from specimens like endo-tracheal aspirate, pus, urine, blood, sputum, pleural fluid and ascitic fluid of patients admitted to different wards and OPDs. The *A. baumannii* and *P. aeruginosa* were identified by using standard bacteriological techniques.^[4] All these isolates were screened for imipenem and meropenem resistance by Kirby-Bauer disk diffusion method and Minimal Inhibitory Concentration (MIC) methods according to CLSI guidelines.^[5]

Extended Spectrum β Lactamase Detection (ESBL)

Combined Disc Diffusion test

This test was carried out for all the screened positive isolates against Ceftazidime (30 μ g) disc with and without Clavulanic acid (10 μ g). A ≥ 5 mm increase in zone diameter of ceftazidime + clavulanic acid versus ceftazidime alone confirms ESBL production.^[5]

Detection of AmpC β -lactamase

Boronic Acid Disc Tests (Screening Test)

Two disc containing 30 μ g ceftazidime were placed on the plate and 20 μ l boronic acid (containing 400 μ g) was

dispensed to one of the ceftazidime disc. Plates were incubated overnight at 35°C aerobically. The test was considered positive when the diameter of the growth-inhibitory zone around ceftazidime disc with boronic acid was 5 mm larger than that around a disk containing the ceftazidime disc alone.^[6]

Detection of Carbapenemases

The detection was done by using Modified Hodge Test (MHT) in which an overnight culture suspension of *Escherichia coli* ATCC 25922 adjusted to 0.5 McFarland standard was inoculated on MHA agar plate (HI-MEDIA, Mumbai, India) and allowed to dry for 3-5min. After drying 10 μ g imipenem disc (HI-MEDIA, Mumbai, India) was placed at the centre of the plate and the test strain was streaked from the edge of the disc to the periphery of the plate in a straight line. Plate was incubated overnight at 35°C aerobically for overnight. Presence of "Clover leaf" type indentation at the intersection of the test organism and the *E. coli* 25922, within the zone of inhibition of the imipenem susceptibility disc was considered positive for carbapenemase production.^[7]

Detection of class A carbapenemase

Phenotypic assays for the identification of KPC and other class A carbapenemases are based on the inhibitory effect of boronic acids, usually (3-aminophenylboronic acid i.e., APB) with either meropenem or imipenem. A cut-off values ≥ 5 mm of zone diameter differences between discs with a carbapenem plus APB and the carbapenem alone is proposed as being indicative of production of KPC or other class A carbapenemase.^[8]

Detection of metallo β -lactamases (MBL)

All the carbapenem resistant isolates were screened for the presence of MBLs by double-disk synergy test (DDST) and combined-disk test (CDT), with Imipenem-EDTA disc.

- i) **Combined-disk Test (CDT):** A suspension of the organism to be tested was prepared in sterile normal saline and its turbidity was matched with 0.5 McFarland standard. A sterile swab dipped into the inoculum was swabbed over MHA plate. Two 10 μ g imipenem discs were placed on the plate and 5 μ l EDTA (750 μ g per disc) stock solution was added to one of the imipenem disc. Plates were incubated overnight at 35°C aerobically. Isolates were identified as metallo β -lactamase positive if the increase in the inhibition zone with the imipenem and EDTA disc was ≥ 7 mm than imipenem disc alone.^[9]
- ii) **Double Disk Synergy Test (DDST):** MHA plate was inoculated as described in CDT, then an IMP disk (10 μ g) was placed near a blank filter paper disk at a centre to centre distance of 10 to 25 mm. 5 μ l of 0.5 M EDTA was applied to the blank disk (750 μ g). After

incubation for 16-18 h, the presence of an enlarged zone of inhibition was interpreted as EDTA synergy test positive.^[7]

Molecular detection of NDM-1 gene

PCR was performed to all the isolates which were confirmed phenotypically to be positive for MBL production. For partial gene PCR amplification, primers specific for *bla*_{NDM-1} using oligonucleotide sequence *bla*_{NDM-1-F}

5'-GGGCAGTCGCTTCCAACGGT-3' and *bla*_{NDM-1-R} 5'-GTAGTGCTCAGTGTCGGCAT-3' was used for reaction with bacterial DNA as template. DNA was extracted by classical method^[3].

Reaction condition kept for amplification were same as used by Manoharan et al with the annealing temperature of 55°C.^[10] Presence of bands of molecular weight of 475bp suggests the presence of *bla*_{NDM-1} gene on gel electrophoresis.

Result

Antimicrobial Susceptibility Testing: (Kirby Bauer disc diffusion Method) : All the 363 isolates were subjected to commonly used antibiotics and results are shown in the table 1.

Mic Study: was performed on all 363 isolates for carbapenems (imipenem and meropenem), Out of which, 130 (36%) isolates showed resistance to imipenem and 121(33%) to meropenem (isolates showing intermediate MIC value were considered resistant) (Table 2).

ESBL and AmpC β – lactamase status among test isolates

Out of 363 isolates of nonfermenters, 55 (15.2%) were ESBL producer, of which 18(9.9%) were *A. baumannii*

and 37(20.3%) were *P. aeruginosa*. Similarly, 57(15.7%) showed AmpC β –lactamases production, of which 36(19.9%) were *A. baumannii* and 21(11.5%) were *P. aeruginosa* isolates.

Carbapenemases Production by The Nonfermenters

Isolated: Of the 130 isolates showing imipenem resistance by both Kirby – Bauer method and MIC study, 41 (32%) isolates showed positive for carbapenemase production by modified Hodge test (Table 3).

Metallo β-lactamases status among test isolates

Of the 130 imipenem resistant isolates tested, 65 (50%) were confirmed to be MBL producers by imipenem- EDTA combined disc method and 56 (43%) were positive for MBL by double disc synergy test (Table 3).

Class A carbapenemases status

Imipenem-Boronic acid disc test was used to screen for the presence of KPC and other class A carbapenemases among all 130 imipenem resistant isolates. A total of 43 (33%) isolates were found to produce class A carbapenemases (Table 3).

Co-production of different class of β-lactamases

Of the 43 MBL positive isolates of *A. baumannii*, 12 (28%) showed co-production of MBL+ESBL, 7(16%) MBL+AmpC and 3(7%) MBL+ESBL+AmpC. Similarly of the 22 MBL positive isolates of *P. aeruginosa*, 9(41%) showed co-production of MBL+ESBL, 4(18%) MBL+AmpC and 4(18%) MBL+ESBL+AmpC.

PCR: was performed using primers specific for *bla*_{NDM-1} gene among 65 MBL producing isolates, *bla*_{NDM-1} could be detected in 13 strains and results are shown in table 4.

Table 1: Antimicrobial susceptibility testing of *A. baumannii* and *P. aeruginosa* by disc diffusion method.

Antibiotic (disc conc. in µg)	<i>A. baumannii</i> (n=181, exception in*)		<i>P. aeruginosa</i> (n=182, exception in*)		Total (n= 363)	
	Number of susceptible isolates	% of susceptible isolates	Number of susceptible isolates	% of susceptible isolates	Number of susceptible isolates	% of susceptible isolates
Carbencillin	19	10.5	121	66.5	140	36.6
Ceftazidime	17	9.4	91	50.0	108	29.8
Gentamicin	34	18.8	84	46.2	118	32.5
Amikacin	44	24.3	88	48.4	132	36.4
Ciprofloxacin	21*(n=138)	15.2	68*(n=120)	56.7	89*(n=258)	34.5
Levofloxacin	64*(n=138)	46.4	65*(n=120)	54.2	129*(n=258)	50.0
Norfloxacin	12*(n=43)	27.3	17*(n=62)	27.4	29*(n=105)	27.6

Antibiotic (disc conc. in µg)	A. baumannii (n=181, exception in*)		P. aeruginosa (n=182, exception in*)		Total (n= 363)	
	Number of susceptible isolates	% of susceptible isolates	Number of susceptible isolates	% of susceptible isolates	Number of susceptible isolates	% of susceptible isolates
Cefepime	13	7.2	27	14.8	40	11.0
Imipenem	93	51.4	148	81.3	241	66.4
Meropenem	136	75.1	120	65.9	256	70.5
Piperacillin/ Tazobactam	88	48.6	99	54.4	187	51.5
Polymyxin-B	132	72.9	152	85.5	284	78.2
Tigecycline	135	74.6	74	40.7	209	57.6

*Norfloxacin was used in isolates from urine specimen while for isolates from other samples ciprofloxacin and levofloxacin were used.

Table 2: Minimum inhibitory concentration (MIC) of clinical isolates A. baumannii and P. aeruginosa against imipenem and meropenem.

Organism	Sensitive		Intermediate		Resistant	
	Imi	Mero	Imi	Mero	Imi	Mero
A. baumannii (n=181)	89 (49.2%)	127 (72.2%)	7 (3.9%)	3 (1.7%)	85 (46.9%)	51 (28.2%)
P. aeruginosa (n=182)	144 (79.1%)	115 (63.2%)	5 (2.7%)	6 (3.3%)	33 (18.1%)	61 (33.5%)
Total	233 (64.2%)	242 (66.7%)	12 (3.3%)	9 (2.5%)	118 (32.5%)	112 (30.8%)

Imi = Imipenem, Mero = Meropenem

For both A. baumannii and P. aeruginosa : Imi(R) Vs Mero(R), $p < 0.001$

Table 3: Results of Modified Hodge test, MBL and Class-A carbapenemases positive isolates among imipenem resistant isolates.

Organism	Carbapenemases detection		MBL producer				Class A carbapenemase producer	
	MHT		CDT		DDST		CDT	
	No.	Percentage of positive	No.	Percentage of positive	No.	Percentage of positive	No.	Percentage of positive
Acinetobacter baumannii (n=92)	24	26.1	43	46.7	37	40.2	22	23.9
Pseudomonas aeruginosa (n=38)	17	44.7	22	57.9	19	50.0	21	55.3
Total= 130	41	31.5	65	50.0	56	43.1	43	33.1

N= number of positive organisms, %= percentage of positive organisms

Table 4: Detection of NDM-1 gene among in different MBL producing isolates of A. baumannii and P. aeruginosa.

Organism	Phenotypically MBL positive isolates	NDM-1	
		N	%
Acinetobacter baumannii	43	7	16.3
Pseudomonas aeruginosa	22	6	27.3
Total	65	13	20.0

N= number of positive organisms, %= percentage of positive organisms

Discussion

In the present study, the two nonfermenters (*A. baumannii* and *P. aeruginosa*) showed highest susceptibility to polymyxin-B (78.2%) followed by meropenem (70.5%), imipenem (66.4%), tigecyclin (57.6%) and combination of piperacillin-tazobactam (51.5%) by using disc diffusion method. Carbapenems which include imipenem, meropenem, ertapenem, doripenem etc., are broad spectrum antibiotics having β -lactam nucleus. These molecules are active against gram positive, gram negative and anaerobic bacteria. Apart from the decrease membrane permeability and activation of the efflux pump in nonfermenters, production of β -lactamases including carbapenemase enzymes are also known to be responsible for conferring the resistance. Carbapenemases have been classified into class A to D. Amongst them, the class B enzymes are clinically most important. This Metallo beta lactamases (MBL) causing drug resistance has been reported worldwide but commonly in Enterobacteriaceae group of bacteria. [11]

However when all the 363 isolates of above two nonfermenters were subjected to determination of MIC value, 35.8% of the isolates was found resistant to the imipenem while 33.3% of isolates were found resistant to meropenem. These rates are almost comparable with that of the disc diffusion method (Imi, 34.1% and Mero, 30.0%) (p value > 0.05). Production of class A carbapenemases could be observed in 11.8% (43/363) of the isolates. Although 17.9% (65/363) of the isolates were found producing Metallo- β -lactamase, only 3.9% (13/363) of the total isolates were found to be producing bla_{NDM-1} gene. Our study is one of the few reports showing presence MBL including bla_{NDM} in *A. baumannii* and *P. aeruginosa*. *A. baumannii* has been reported producing bla_{NDM-1} from south India, [12] as well as from other parts of the world like Germany, [13] Egypt, [14] China. [15] However, there is one report from Serbia showing presence of NDM in *P. aeruginosa*. [16]

Interestingly, imipenem was found to be less effective for *A. baumannii* as compared to meropenem (p value <0.01). Contrary to this for *P. aeruginosa* imipenem was found to be better drug as compared to meropenem (p value <0.01). This difference may be explained on the basis of differential sensitivity to β -lactamases and orp-gene expression for imipenem and meropenem. In case of *P. aeruginosa* which has stronger efflux pump activity than *A. baumannii*, imipenem may be better as meropenem has been stated to act as a substrate for Mex AB-*oprM* efflux pump because of the presence of hydrophobic side chain at position 2 whereas imipenem containing strongly charged

hydrophilic side chains cannot become a substrate for this efflux pump. [17] However, contrary to this, there is a report from France showing meropenem as the better drug for *P. aeruginosa* and imipenem for *A. baumannii*. [18]

Conclusion

Treatment of infections caused by nonfermenters like *P. aeruginosa* and *A. baumannii* producing different carbapenemases including NDM-1 are now posing serious challenge as these infections are resistant to almost all available antimicrobial agents. Now it is the time that uniform antibiotic policy should be implemented through WHO so that the unethical and irresponsible marketing practices by pharmaceutical companies may be stopped. The up to date microbiological services should be available even in developing world. These steps might help us in retaining the relevance of some newer drugs e.g. tigecycline, colistin, polymyxin and aztreonam etc. Moreover, we must explore the alternatives of the antibiotics now.

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Competing Interests

None Declared

Reference

1. Fass RJ, Barnishan J, Solomon MC, Ayers LW. In vitro activities of quinolones, β -lactams, tobramycin and trimethoprim sulfamethoxazole against nonfermentative gram negative bacilli. Antimicrob. Agents. Chemother. 1996; 40: 1412-18.
2. Gladstone P, Rajendra P, Brahmtham KN. Incidence of carbapenem resistant nonfermenting gram negative bacilli from patients with respiratory infections in the intensive care units. Indi J Med Microbiol. 2005; 23: 189-91.
3. Fritsche TR, Sader HS, Toleman MA, Walsh TR, Jones RN. Emerging metallo-beta-lactamase-mediated resistances: a summary report from the worldwide SENTRY antimicrobial surveillance program. Clin Infect Dis. 2005; 41: 276 -278.
4. Collee JG, Miles RS, Wan B. Test for identification of bacteria. In: Collee JG, Marmion BP, Fraser AG, Simmons A, editors. Mackie & McCartney Practical Medical Microbiology. 14th edn. Edinburgh: Churchill Livingstone. 1996; 131-150.
5. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility

- testing; Clinical and Laboratory Standards Institute Wayne; (2014); 23rd Informational Supplement M100-S24
6. Yagi T, Wachino J, Kurokawa H, Suzuki S, Yamara K, Doi Y, Shibata N, Kato H, Shibayama K, and Arakawa Y. Practical methods using boronic acid compounds for identification of class C β -lactamase-producing *Klebsiella pneumoniae* and *Escherichia coli*. *J Clin Microbiol*. 2005; 43: 2551-58.
 7. Lee K, Chong Y, Shin HB, Kim YA, Yong D, Yum JH. Modified Hodge and EDTA-disk synergy tests to screen metallo-beta-lactamase-producing strains of *Pseudomonas* and *Acinetobacter* species. *Clin Microbiol Infect*. 2001; 7: 88-91.
 8. Pasteran F, Mendez T, Guerriero L, Rapoport M, Corso A. Sensitive screening tests for suspected class A carbapenemase production in species of *Enterobacteriaceae*. *J Clin Microbiol*. 2009; 47: 1631-39.
 9. Yong D, Lee K, Yum JH, Shin HB, Rossolini GM, Chong Y. Imipenem-EDTA disk method for differentiation of metallo-beta-lactamase-producing clinical isolates of *Pseudomonas* spp. and *Acinetobacter* spp. *J Clin Microbiol*. 2002; 40: 3798-3801.
 10. Manoharan A, Chatterjee S, Mathai D. Detection of Metallo beta lactamases producing *Pseudomonas aeruginosa*. *Indian J Med Microbio*. 2010; 28: 241-44.
 11. Nordmann P, Carrer A. Carbapenemases in *enterobacteriaceae*. *Arch Pediatr*. 2010; 17(4): 154-62.
 12. Kumarasamy KK, Toleman MA, Walsh TR, Bagaria J, Butt F, Balakrishnan R et al. Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *Lancet Infect Dis*. 2010; 10: 597-602.
 13. Pfeifer Y, Wilharm G, Zander E, Wichelhaus TA, Gottig S, Hunfeld KP et al. Molecular characterization of blaNDM-1 in an *Acinetobacter baumannii* strain isolated in Germany in 2007. *J Antimicrob Chemother*. 2011; 66: 1998-2001.
 14. Kaase M, Nordmann P, Wichelhaus T A, Gatermann S G, Bonnin RA, Poirel L. NDM-2 carbapenemase in *Acinetobacter baumannii* from Egypt. *J. Antimicrob. Chemother*. 2011; 66: 1260-62.
 15. Chen Y, Zhou Z, Jiang Y, Yu Y. Emergence of NDM-1 producing *Acinetobacter baumannii* in China. *J. Antimicrob. Chemother*. 2011; 66: 1255-59.
 16. Jovicic B, Lepsanovic Z, Suljagic V, Rackov G, Begovic J, Topisirovic L, et al. Emergence of NDM-1 Metallo- β -Lactamase in *Pseudomonas aeruginosa* Clinical Isolates from Serbia. *Antimicrobial agents and chemotherapy*. 2011; 55: 3929-31.
 17. Lee A, Mao W, Warren MS, Mistry A, Hoshino K, Okumara R et al. Interplay between Efflux Pumps May Provide Either Additive or Multiplicative Effects on Drug Resistance. *Journal of bacteriology* . 2000; 182(11): 3142-50
 18. Guillou MLJ, Kempf M, Cavallo JD, Chomarar M, Dubreuil L, Maugein J, et al. Comparative in vitro activity of Meropenem, Imipenem and Piperacillin/tazobactam against 1071 clinical isolates using 2 different methods: A French multicentre study, *BMC Infectious Diseases*. 2010; 10:72.
 19. Walsh TR. Clinically significant carbapenemases: An update. *Curr Opin Infect Dis*. 2008; 21: 367-71.