

Phenotypic characterization and colistin susceptibilities of carbapenem-resistant of *Pseudomonas aeruginosa* and *Acinetobacter* spp.

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Abstract

Introduction: *Pseudomonas aeruginosa* and *Acinetobacter* spp. are important nosocomial pathogens and carbapenem resistance is an emerging threat. Therapeutic options for infections with these isolates include colistin. This study was conducted to determine the prevalence of carbapenem resistance in *P. aeruginosa* and *Acinetobacter* spp. bloodstream isolates, phenotypically characterize the resistance mechanisms and evaluate the *invitro* activity of colistin.

Methodology: Consecutive 145 (95 *P.aeruginosa* and 50 *Acinetobacter* spp.) non-repeat isolates were included. Antibiotic susceptibility testing was performed per CLSI guidelines. MIC for carbapenems and colistin was performed using Etest. Isolates showing reduced susceptibility or resistance to the carbapenems were tested for metallo- β -lactamase (MBL) production using imipenem-EDTA combined disk and MBL Etest.

Results: Carbapenem resistance was observed in 40% *P. aeruginosa* and 66.0% *Acinetobacter* spp. Carbapenem-resistant (CA-R) isolates were significantly ($p < 0.05$) more frequently resistant to the other antibiotics than carbapenem-susceptible isolates. Approximately half of the CA-R strains were multidrug-resistant, and 3.1-5.5% were resistant to all antibiotics tested. MBL was found in 76.3% and 69.7% of the *P. aeruginosa* and *Acinetobacter* spp., respectively. Colistin resistance was observed in three (6.0%) *Acinetobacter* isolates and eight (8.4%) *P. aeruginosa*. MIC₅₀ for carbapenems were two to four times higher for MBL-positive compared to MBL-negative isolates, but no difference was seen in MIC for colistin.

Conclusion: Carbapenem resistance was observed to be mediated by MBL in a considerable number of isolates. Colistin is an alternative for infections caused by CA-R isolates; however, MIC testing should be performed whenever clinical use of colistin is considered.

Key words: carbapenem resistance; *Pseudomonas aeruginosa*; *Acinetobacter* spp.; metallo- β -lactamase; colistin susceptibility and MIC

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Introduction

Non-fermenting bacilli such as *Pseudomonas aeruginosa* and *Acinetobacter* spp. are pathogens emerging as frequent causes of nosocomial infections, especially pneumonia and sepsis, with mortality rates of 27-48% in critically ill patients [1,2]. Management is difficult, as the strains often display intrinsic and acquired resistance to multiple classes of antibiotics, including extended-spectrum cephalosporins, aminoglycosides, and fluoroquinolones, severely limiting therapeutic options [3]. The introduction of carbapenems meropenem (MEM) and imipenem (IPM) into clinical practice was a great advance in the treatment of serious infections caused by these multidrug-resistant (MDR) bacteria; carbapenems are often employed as drugs of last resort in these bacteria. However, the emergence and increasing frequency of isolation of carbapenem-resistant (CA-R) *P.*

aeruginosa and *Acinetobacter* spp. is a cause for concern [4].

Data of the antibiotic susceptibilities of *Acinetobacter* spp. from different geographical regions reveal that the resistance of *Acinetobacter* to IPM was in the range of no resistance to 40% (2000-2004) [5], which has subsequently risen to 85-87% (2007) in the ICUs of European countries such as Greece, and in the USA [6], while resistance to MEM has risen from 18% (1997-2000) to 43.4% (2006) as reported from multicentric data by the MYSTIC program [7,8]. A similar trend in *P. aeruginosa* has been reflected in a Brazilian study, where rates of resistance to IPM increased from 6.06% in 2004 to 45.09% in 2008; resistance to MEM increased from 6.89 % to 20.0% during the same period [9]. In India, carbapenem resistance ranges from 10.9- 69% [10, 11] in *P. aeruginosa*; a range of 9.1-100% [12,13] in *Acinetobacter* spp. has been reported in various patient

populations in different sample types, mostly consisting of respiratory specimens and wound swab samples. To our knowledge, only a single Indian study [14] has reported carbapenem resistance (50%) in *Acinetobacter baumannii* isolated from blood of neonates with sepsis.

Of the various mechanisms of carbapenem resistance in *P. aeruginosa* and *Acinetobacter* spp., namely impermeability arising via the loss of outer membrane porins, the upregulation of an active efflux pump (MexAB-OprM system, AdeABC pump) and production of metallo-beta-lactamases (MBLs) that mediated by the MBLs is of great concern [15-17]. The genes responsible for the production of MBLs are typically part of an integron structure carried on transferable plasmids; hence, isolates producing MBLs are often resistant to different groups of antimicrobial agents, which can be transferred to various types of bacteria [17]. Other than MBLs, derepressed AmpC β -lactamases and OXA-carbapenemases are also an increasingly important source of carbapenem resistance in these two organisms [15-17].

The emergence of CA-R *P. aeruginosa* and *Acinetobacter* spp. has led to the reconsideration of colistin, a polymyxin antibiotic that has activity against Gram-negative organisms including *Pseudomonas* and *Acinetobacter* spp. [18]. The increased clinical use of parenteral polymyxins has created a pressing need for up-to-date susceptibility data and standardized susceptibility testing methods in these two pathogens. In addition, it is essential to rapidly screen and detect MBLs in *P. aeruginosa* and *Acinetobacter* spp., which could help to modify therapy and to initiate effective infection control to prevent further dissemination. Numerous Indian studies have documented the presence of MBLs in *P. aeruginosa* [19-22], but only a few [13,21,22] have documented the same in *Acinetobacter* spp. Further, the issue of colistin susceptibility in these two organisms has not been addressed sufficiently [20, 23].

The present study was conducted to determine the prevalence of carbapenem resistance in *P. aeruginosa* and *Acinetobacter* spp. bloodstream isolates in our hospital, to phenotypically characterize the resistance mechanisms, and to evaluate the *in vitro* activity of colistin against the isolates.

Methodology

The study was conducted at the Department of Microbiology, Vardhman Mahavir Medical College and Safdarjung Hospital, a 1500-bed tertiary care, referral, and teaching hospital in North India over a six-month period between January and June 2011.

Bacterial isolates

A total of 145 (95 *P. aeruginosa* and 50 *Acinetobacter* spp.) non-repeat, non-duplicate bloodstream isolates recovered from patients admitted to various wards of the hospital were included in the study. The organisms were identified by conventional biochemical tests using standard microbiological techniques [24].

Antimicrobial susceptibility testing and MIC determination

Antimicrobial susceptibility testing and interpretation was performed for ten different therapeutically relevant antibiotics on Mueller Hinton agar (HiMedia, Mumbai, India) by the standard disk diffusion method according to Clinical Laboratory Standards Institute (CLSI, 2011) guidelines [25]. The antibiotics tested (disk concentrations in μg) were as follows: ceftazidime (30), piperacillin (100), amikacin (30), netilmicin (30), gentamicin (10), ciprofloxacin (5), piperacillin/tazobactam (100/10), cefoperazone/sulbactam (75/10), imipenem (10), and meropenem (10). All the disks were procured from HiMedia, Mumbai, India. The tests were quality controlled using *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 29213. Organisms “intermediate” were included in the percentage of resistant isolates.

Minimum inhibitory concentration (MIC) was determined for all isolates to IPM, MEM, and colistin by the Etest (AB Biodisk, Solna, Sweden). The MIC ($\mu\text{g}/\text{ml}$) breakpoints for IPM and MEM for both the organisms were defined as follows: sensitive (≤ 4), intermediate (8), and resistant (≥ 16) [25]. For colistin, the MIC ($\mu\text{g}/\text{ml}$) breakpoints for *P. aeruginosa* were defined as sensitive (≤ 2), intermediate (4), and resistant (≥ 8); breakpoints for *Acinetobacter* spp. were defined as sensitive (≤ 2) and resistant (≥ 4) only [25]. For carbapenems, where both disk diffusion and MIC testing was performed, the results of the MIC test were taken for final consideration of the susceptibility status of the organisms.

Figure 1: Antibiotic –resistance profiles **1A** MBL-positive (n=29) and MBL –negative (n=9) *P. aeruginosa* and **1B** MBL-positive (n=23) and MBL –negative (n=10) *Acinetobacter* spp.

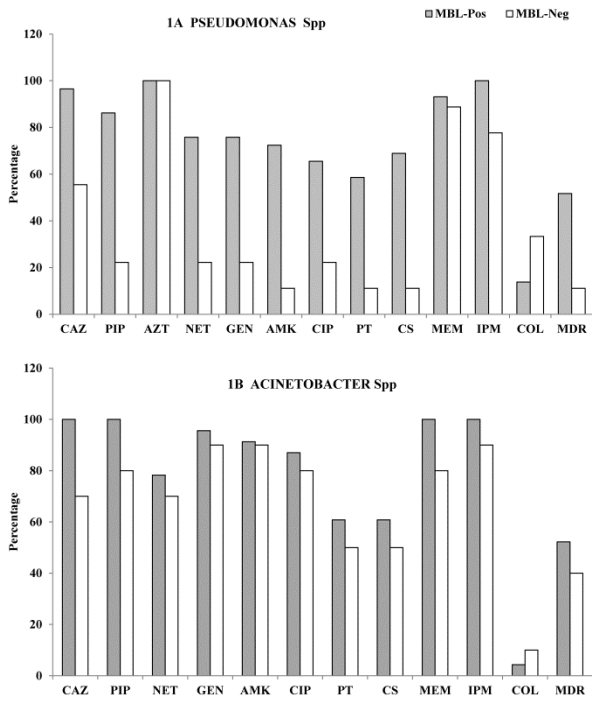
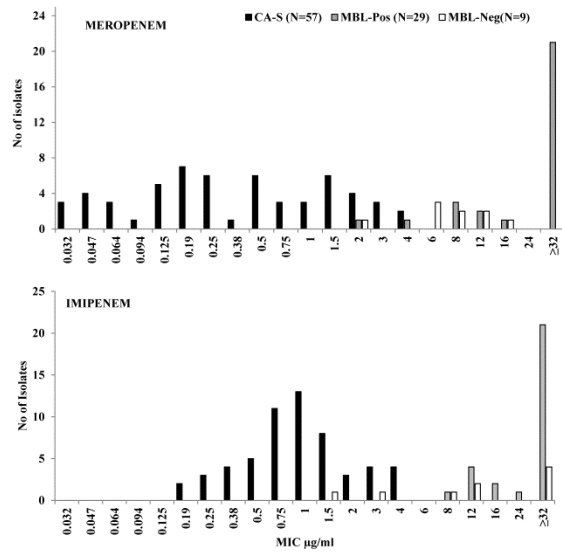
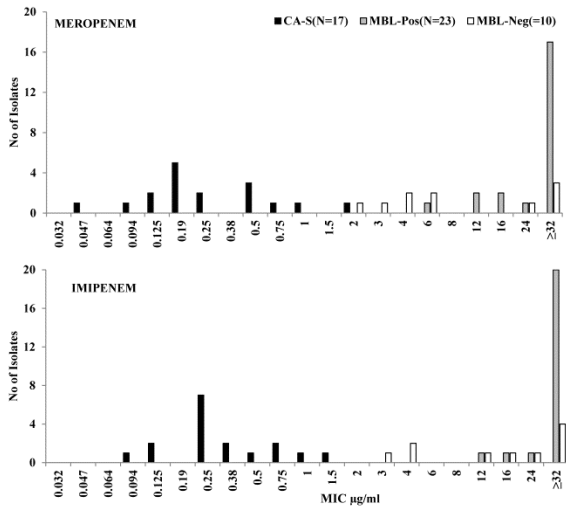


Figure 2A: Minimum inhibitory concentration (MIC), MIC₅₀, MIC₉₀ of *P. aeruginosa* (n=95) to carbapenems



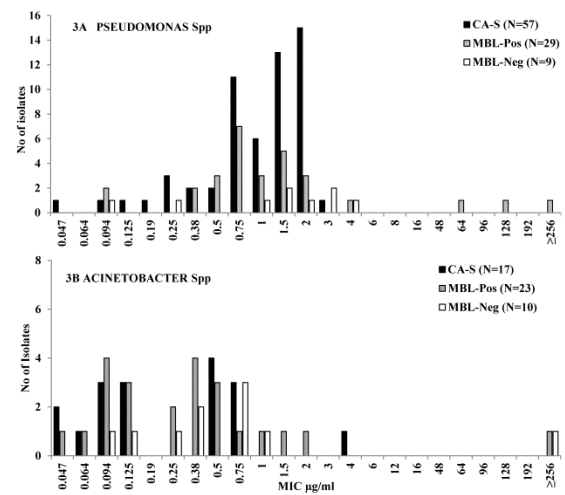
Antibiotic	<i>Pseudomonas</i> spp					
	Carbapenem Sensitive		Carbapenem – Resistant			
			MBL- Positive		MBL- Negative	
	MIC 50	MIC 90	MIC 50	MIC 90	MIC 50	MIC 90
MEROPENEM	0.19	1.5	>32	>32	7	12
IMIPENEM	0.75	2	>32	>32	12	>32

Figure 2B: Minimum inhibitory concentration (MIC), MIC₅₀, MIC₉₀ of *Acinetobacter* spp. (n=50) to carbapenems



Antibiotic	<i>Acinetobacter</i> spp					
	Carbapenem Sensitive		Carbapenem – Resistant			
			MBL- Positive		MBL- Negative	
	MIC 50	MIC 90	MIC 50	MIC 90	MIC 50	MIC 90
MEROPENEM	0.19	0.75	>32	>32	6	>32
IMIPENEM	0.25	0.75	>32	>32	16	>32

Figure 3: Minimum inhibitory concentration (MIC), MIC₅₀, MIC₉₀ of **3A** : *Pseudomonas aeruginosa* (n=95) and **3B**: *Acinetobacter* spp. (n=50) to Colistin



Antibiotic	COLISTIN MIC µg/ml					
	Carbapenem Sensitive		Carbapenem – Resistant			
			MBL- Positive		MBL- Negative	
	MIC 50	MIC 90	MIC 50	MIC 90	MIC 50	MIC 90
PSEUDOMONAS	1.25	2	0.87	4	1.5	3
ACINETOBACTER	0.125	0.75	0.31	1.5	0.38	1

Carbapenem-resistant isolates were defined as those intermediate or resistant to any one of the carbapenems, (MEM and/or IPM). Multidrug resistance was defined as non-susceptibility to at least one agent in three or more antimicrobial categories [26].

Metallo-β-lactamase detection

Tests for MBL production were performed on isolates showing reduced susceptibility or resistance to the carbapenems [25] (IPM zone diameter ≤ 15 mm and/or MIC ≥ 2 $\mu\text{g/ml}$), and/or MEM zone diameter ≤ 15 mm and/or MIC ≥ 2 $\mu\text{g/ml}$) by two methods, the imipenem-EDTA combined disk method and the imipenem/imipenem-EDTA MBL Etest. The imipenem -EDTA combined disk method was performed as described by Yong *et al.* [27], where a 750 μg EDTA disk was used in combination with an IPM disk along with a single IPM disk. A zone difference of ≥ 7 mm between IPM alone and with EDTA was considered MBL-positive. In the MBL Etest (AB Biodisk, Solna, Sweden), the presence of MBL was reflected by a reduction in the IPM MIC in the presence of EDTA of greater than or equal to eight-fold (IP/IPI ≥ 8), by the observation of a “phantom zone,” or a deformation of IPM inhibition ellipse per the manufacturer’s instructions.

Results and Discussion

The unit-wise distribution of the *P. aeruginosa* (n = 95) isolates was as follows: intensive care units (n = 48; 50.5%), paediatric including neonatal unit (n = 35; 36.8%), and adult medical unit (n = 12; 12.6%). Similarly, the unit-wise distribution of the *Acinetobacter* spp. (n = 50) isolates was: intensive care units (n = 35; 70.0%), paediatric including neonatal unit (n = 9; 18.0%), and adult medical unit (n = 6; 12.0%). Overall resistance of the isolates (*P. aeruginosa* vs *Acinetobacter* spp.) to the antibiotics tested were as follows: ceftazidime (57.9% v. 84.0%), piperacillin (37.9% v. 94.0%), netilmicin (36.8% v. 58.0%), gentamicin (40.0% v. 80.0%), amikacin (33.7% v. 72.0%), ciprofloxacin (35.8% v. 64.0%), piperacillin/tazobactam (22.1% v. 42.0%), cefoperazone/sulbactam (24.2% v. 42.0%), meropenem (36.8% v. 62.0%), imipenem (37.9% v. 64.0%) and colistin (8.4% v. 6.0%) (Tables 1A and 1B). Multidrug resistance was observed in 44 (46.3%) and 36 (72.0%) of the *P. aeruginosa* and *Acinetobacter* spp. isolates, respectively.

A total of 38 (40%) of *P. aeruginosa* and 33 (66.0%) of *Acinetobacter* spp. were observed to be CA-

R. Remarkably, CA-R isolates were found to be significantly ($p < 0.05$) more frequently resistant to the other antibiotics than carbapenem-susceptible isolates were (Tables 1A and 1B). Furthermore, close to half of the CA-R strains of both organisms were MDR, and 3.1-5.5% of them were resistant to all antibiotics tested. Metallo- β -lactamase production was found in 29 (76.3%) and 23 (69.7%) of the CA-R *P. aeruginosa* and *Acinetobacter* spp. isolates, respectively. In comparison to the disk method, the Etest detected one and three additional isolates of *P. aeruginosa* and *Acinetobacter* spp. to be MBL producers. A comparison of antibiotic susceptibility profiles of CA-R MBL-positive and MBL-negative isolates in both the organism groups showed a higher prevalence of resistance in MBL-positive isolates as compared to the MBL-negative ones for all antibiotics except colistin (Figure 1A and 1B). Since MBLs are carried on plasmids, this may explain the higher prevalence of co-resistance to other antibiotics found in MBL-positive isolates.

The minimum inhibitory concentration of *Pseudomonas aeruginosa* and *Acinetobacter* spp. to carbapenems are shown in Figure 2A and 2B, respectively. High-level MEM and IPM resistance (MIC ≥ 16 $\mu\text{g/ml}$) was noted in 23 (60.5%) and 28 (73.7%) of the CA-R isolates of *P. aeruginosa* (Figure. 2A). Similarly, for *Acinetobacter* spp., high-level MEM and IPM resistance was noted in 24 (72.7%) and 29 (87.9%) of the carbapenem-resistant isolates (Figure 2B). Amongst CA-R isolates, MIC₅₀ for both MEM and IMP were two to four times higher for MBL producers compared to MBL non-producers (Figure 2A and 2B) in both organism groups. Colistin resistance was observed in 3 (6.0%) *Acinetobacter* isolates, which included two carbapenem-resistant (2/33, 6.1%) and one (1/17, 5.9%) carbapenem-susceptible isolate. In the case of *P. aeruginosa*, colistin resistance was observed in eight (8.4%) isolates, including seven (7/38, 18.4%) carbapenem-resistant and one (1/57, 1.8%) carbapenem-susceptible isolate. (Tables 1A & 1B). MIC range, MIC₅₀ and MIC₉₀ of *P. aeruginosa* and *Acinetobacter* spp. to colistin are shown in Figure 3A and 3B. There was no significant difference in MIC₅₀ and MIC₉₀ amongst carbapenem-susceptible, CA-R MBL- positive and CA-R MBL-negative isolates of *P. aeruginosa* and *Acinetobacter* spp.

Table 1A. Antimicrobial susceptibility pattern of *P. aeruginosa* (n=95) bloodstream isolates in a tertiary care hospital

Antibiotic or resistance phenotype	No. (%) of resistant isolates among:		
	Total (n=95)	Carbapenem-susceptible isolates (n=57)	Carbapenem-resistant isolates (n=38)
Ceftazidime	55 (57.9)	22 (38.6)	33 (86.8) *
Piperacillin	36 (37.9)	9 (15.8)	27 (71.0) *
Netilmicin	35 (36.8)	11 (19.3)	24 (63.1) *
Gentamicin	38 (40.0)	14 (24.6)	24 (63.1) *
Amikacin	32 (33.7)	10 (17.5)	22 (57.9) *
Ciprofloxacin	34 (35.8)	13 (22.8)	21 (55.3) *
Piperacillin/tazobactam	21 (22.1)	3 (5.3)	18 (47.3) *
Cefoperazone/sulbactam	23 (24.2)	2 (3.5)	21 (55.26) *
Meropenem #	35 (36.8)	0 (0.0)	35 (92.1) *
Imipenem #	36 (37.9)	0 (0.0)	36 (94.7) *
Colistin	8 (8.4)	1 (1.7)	7 (18.4) *

33 isolates resistant to both Meropenem and Imipenem, 3 isolates sensitive to Meropenem but resistant to Imipenem, and 2 isolates sensitive to Imipenem but resistant to Meropenem .

* p < 0.05 (=significant) for difference in resistance between carbapenem-resistant and -susceptible isolates by chi-square test or Fisher’s exact test wherever applicable

Table 1B. Antimicrobial susceptibility pattern of *Acinetobacter* spp. (n=50) bloodstream isolates in a tertiary care hospital

Antibiotic or resistance phenotype	No. (%) of resistant isolates among:		
	Total (n=50)	Carbapenem-susceptible isolates (n=17)	Carbapenem-resistant isolates (n=33)
Ceftazidime	42 (84.0)	12 (70.5)	30 (90.9)
Piperacillin	47 (94.0)	16 (94.1)	31 (93.9)
Netilmicin	29 (58.0)	4 (23.5)	25 (75.7) *
Gentamicin	40 (80.0)	9 (52.9)	31 (93.9) *
Amikacin	36 (72.0)	6 (35.3)	30 (90.9) *
Ciprofloxacin	32 (64.0)	4 (23.5)	28 (84.8) *
Piperacillin/tazobactam	21 (42.0)	2 (11.8)	19 (57.5) *
Cefoperazone/sulbactam	21 (42.0)	2 (11.8)	19 (57.5) *
Meropenem #	31 (62.0)	0 (0.0)	31 (93.9) *
Imipenem #	32 (64.0)	0 (0.0)	32 (96.9) *
Colistin	3 (6.0)	1 (5.9)	2 (6.06)

30 isolates resistant to both Meropenem and Imipenem, 2 isolates sensitive to Meropenem but resistant to Imipenem PM, and 1 isolate sensitive to Imipenem but resistant to Meropenem

* p < 0.05 (=significant) for difference in resistance between carbapenem-resistant and -susceptible isolates by chi-square test or Fisher’s exact test wherever applicable

Thus, a high prevalence of resistance was observed in both *P. aeruginosa* and *Acinetobacter* isolates to the tested antibiotics (except to colistin), including carbapenems, with rates ranging from 22.1-57.9% and 42 -94.0%, respectively. A recent study [28] on selected bacteraemic pathogens from private institutions in South Africa also revealed a high prevalence of antimicrobial resistance in *P. aeruginosa* and *A. baumannii*. Carbapenem resistance

in *P. aeruginosa* was observed to be 42% and 45% for MEM and IPM, while in *A. baumannii* it was 32% and 33% for the two antimicrobial agents. Similarly, resistance to cefepime, piperacillin-tazobactam and fluoroquinolone was 53% v. 43%, 48% v. 42% and 46% v. 31%, respectively [28]. A study on bacteraemic isolates of febrile neutropenic patients in Pakistan [29] showed an increase in IPM resistance from 0% in 1999-2000 to 37% in 2001-2006 in the

non-enterobacteriaceae isolates comprising mostly of *P. aeruginosa* and *Acinetobacter* spp. Similarly, a study in the United Kingdom [30] on *Acinetobacter* BSI isolates found carbapenem resistance rising from 0% in 1998 to 55% in 2006.

A large proportion of the CA-R isolates in our study were found to be MBL producers. In India, MBL-producing *P. aeruginosa* was first reported in 2002 [31]. Since then, the incidence of MBL production by *P. aeruginosa* has been reported to range from 8.05-27.7 % from various clinical specimens across the country [20,22,32]. Specifically, amongst carbapenem-resistant strains, the incidence of MBL production has been found to vary from 50% to 87.5% [19,21], which is similar to the findings of the present study. In *Acinetobacter* spp., the incidence of MBL production has been observed to vary from 0% to 70.9 % amongst the CA-R isolates [13,14,21,22]. A point to note is that most of the studies, including the present study, used the Etest MBL strips or IPM-EDTA combined disk tests for the detection of MBLs, where the positivity may be dependent on the inhibitory effect of EDTA on dimer formation of a carbapenem-hydrolyzing-class D-β-lactamase such as OXA-23 or OXA-24, a predominant mechanism of carbapenem resistance in *Acinetobacter* spp. This could explain the high prevalence of MBL- positive *Acinetobacter* isolates reported in these studies [20,21]. However, no phenotypic tests are currently available to detect the presence of OXA enzymes in *Acinetobacter* spp.

Since only a few systematic surveys of antibiotic resistance have been performed on colistin, reliable data on its true resistance are lacking. Interpretation of categoric resistance has been further complicated by susceptibility criteria, which vary across countries [33]. In a study of 102 bacterial isolates [33], susceptibility to colistin was most prevalent in *Acinetobacter* spp., with no resistant isolates detected, whereas 33% of *P. aeruginosa* isolates were resistant to colistin. A survey of cystic fibrosis patients in the United Kingdom reported that 3.1% of *P. aeruginosa* isolates were resistant to colistin, based on a susceptibility breakpoint concentration $\leq 4 \mu\text{g}/\text{ml}$ [34]. In a study from India, all 20 MBL-producing *P. aeruginosa* isolates were susceptible to colistin, with MIC ranging from 0.5-0.032 $\mu\text{g}/\text{ml}$ [35]. However, in another Indian study [20], colistin resistance was found in 51.7 % of CA-R *P. aeruginosa* strains. In the case of *Acinetobacter* spp., a study from North India [23] reported that 3.5% of the total and 16% of the

CA-R MDR strains were resistant to both colistin and tigecycline.

A limitation of the current study is the lack of genotypic confirmation for the presence of carbapenem-hydrolyzing genes. Studies from India, however, have demonstrated the presence of both OXA enzymes and MBLs by PCR in a considerable number of *P. aeruginosa* and *Acinetobacter* isolates. MBL genes by PCR have been detected in 42% [13], 46.5% [36], and 16.28% [37] of carbapenem-resistant *A. baumannii* isolates in various Indian studies, while both OXA -β-lactamases and MBLs were detected in 91.4% and 46.5% *A. baumannii* isolates in another study [38]. Similarly, the presence of MBL genes by PCR has been reported in 59.0% of *P. aeruginosa* strains [36].

Conclusions

The high prevalence of antibiotic resistance observed in the current study is significant, as it heralds the beginning of the post-antibiotic era where only a few therapeutic options would be available for treatment. Mechanisms other than MBL may contribute to carbapenem resistance; it is necessary to develop methods to detect these. Though colistin may be considered as a treatment alternative in infections caused by carbapenem-resistant *P. aeruginosa* and *Acinetobacter* spp., susceptibility testing (particularly MIC) should be performed whenever clinical use of colistin is considered.

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