Carbapenem Resistance among *Pseudomonas aeruginosa* isolated from Clinical Samples with a Special Reference to Colistin Resistance

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Abstract

BACKGROUND

Infections caused by *Pseudomonas aeruginosa* present a huge treatment challenge due to the high antibiotic resistance. The rapid development of carbapenem resistance is alarming and calls for implementing surveillance measures. This study aimed to detect antibiotic susceptibility of *P. aeruginosa* isolated from different clinical samples and to detect carbapenem resistance, metallo β-lactamase and also colistin resistance among carbapenem-resistant *P. aeruginosa*.

MATERIALS AND METHODS

A cross-sectional study with a sample size of 100 clinical isolates of pathologically significant *P. aeruginosa* collected from patients of all age groups identified by standard methods were included. The prevalence of *P. aeruginosa* was estimated at 7.15% (95/1566) from a previous study. The sample size was estimated at 102 with a 95% confidence level and a margin of error of 5%. Antimicrobial susceptibility testing was done by the standard Kirby-Bauer disc diffusion method. Metallo β-lactamase detection done by the combined disc test and Modified Hodge test. Carbapenemase gene detection was done by Real-time polymerase chain reaction. Susceptibility to colistin was detected by the Vitek-2 system.

RESULTS

Out of 100 *P. aeruginosa* isolates, 52% were from pus, 22% respiratory samples,16% urine and 10% from blood respectively. In antimicrobial susceptibility testing, 30% of the strains were multidrug-resistant, 38% of the strains were sensitive and 32% were intermediate. The highest resistance was observed against fluoroquinolones. Carbapenem resistance was observed as imipenem 17 % and meropenem 16 %. Among the carbapenem-resistant *P. aeruginosa*, 47% of the strains were metallo β-lactamase producers by the phenotypic method. Gene detection for carbapenemase revealed *blaNDM* to be the most common gene carried by 35.2% of the MBL-positive carbapenem-resistant strains. All the carbapenem-resistant strains were susceptible to colistin.

CONCLUSION

Maximum number of *P. aeruginosa* was isolated from pus samples. Prevalence of carbapenem resistance was found to be less in our hospital, with a rate of 17%. The highest resistance was observed against fluoroquinolones. Among carbapenamase, blaNDM seems to be the most prevalent gene at 23%, followed by *blaIMP* at 11%, *blaIMP* and *blaNDM* at 5.8%, and *blaNDM*, *blaIMP* and *blaVIM* at 8 % respectively. No strains exhibited resistance against colistin when tested by the Vitek-2 system.

Keywords: *Pseudomonas aeruginosa, Metallo β-lactamase, Carbapenem resistance, Multidrug Resistance.*

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Introduction

P. aeruginosa is a ubiquitous gram-negative bacillus that can inhabit a broad variety of nutritionally minimal environments, colonise moist surfaces and can grow even in disinfectants, soaps, eye drops, etc (1). With a

large genome spanning over 6 million base pairs, coding 5567 genes, *P. aeruginosa* is highly adaptable (2*).* Its high rate of intrinsic resistance to antimicrobials and disinfectants (3) makes it a common and troublesome pathogen. *P. aeruginosa* is the third most common aetiology for nosocomial infections (4). *Pseudomonas aeruginosa* is commonly associated with respiratory tract infections, including ventilator-associated pneumonia (VAP), as well as urinary tract infections (UTIs), burn wounds, and soft tissue infections (5). Antibiotic resistance mechanisms exhibited by *P. aeruginosa* can be intrinsic or acquired.

In clinical practice, multidrug-resistant *P. aeruginosa* (MDR-PA) is a rapidly evolving clinical challenge often associated with severe morbidity, mortality, and increased economic burden. Carbapenems have long been considered the drugs of choice for treating these infections. However, the widespread emergence of carbapenem resistance in recent years has led to the use of polymyxin B and colistin, despite their known adverse effects, such as nephrotoxicity and neurotoxicity. Alarmingly, *P. aeruginosa* has also begun to develop resistance to colistin. The *mcr* gene that is responsible for colistin resistance has been observed globally in 57 countries (6). This study analysed the prevalence of resistance to carbapenem in *P. aeruginosa* strains from clinical specimens with special regard to resistance to colistin.

Material and Methods

Study design

This was a cross-sectional study conducted in the Department of Microbiology, Kannur Medical College, Kannur, Kerala, from December 2019 to November 2021. The study was undertaken with 100 strains of *P. aeruginosa* isolated from clinical samples in suspected cases of respiratory tract infection, urinary tract infection, skin, and soft tissue infection.

Laboratory procedures

P. aeruginosa isolates were cultured and identified as pathologically significant from different clinical samples collected from patients of all age groups. The bacterial isolates were identified by Gram stain, cultural characteristics, and biochemical reactions, which included oxidase test, acetamide utilisation, arginine dihydrolase test, triple sugar iron test, oxidation fermentation test, citrate utilisation test and indole test (7).

Antibiotic susceptibility testing of all isolates of *P. aeruginosa* was carried out by the standard Kirby-Bauer disc diffusion method on Mueller-Hinton agar (Hi-media, India) as per CLSI 2019. *P. aeruginosa* ATCC 27853 was used as a control (8). To test the antibiotic susceptibility, the following antibiotics were used: 10 µg of Tobramycin, 10 µg of gentamicin, 30µg of amikacin, 30µg of ceftazidime, 30µg of cefepime, 100/10µg of piperacillin-tazobactam, 5µg of ciprofloxacin 5µg of levofloxacin, 10µg of norfloxacin, 10µg of imipenem, 10µg of meropenem, 10µg of aztreonam and 10µg of colistin.

Detection of Carbapenemase by modified Hodge test

P. aeruginosa strains resistant to carbapenems were subjected to the Modified Hodge test. A lawn culture of 1:10 dilution of E. coli ATCC 25922 was done on the Muller-Hinton agar. A 10µg Imipenem disc was placed in the centre. The test organism was streaked in a straight line from the edge of the disc to the edge of the plate. Four strains were tested in each plate, and *Klebsiella pneumoniae* ATCC 1705 was used as an internal control (9). After 24 hours of incubation, the plate was examined for a clover leaf-type indentation at the intersection of the test organism and *E. coli* 25922, within the zone of inhibition of the carbapenem susceptibility disk.

Detection of Carbapenemase by combined disc test

Carbapenem-resistant *P. aeruginosa* strains were also subjected to a combined disc test for the detection of Metallo β-lactamase. A standard suspension of test isolate was inoculated on a Mueller Hinton agar plate, 0.5 M ethylene diamine tetra acetic acid (EDTA)

was prepared, and pH was adjusted to 8.2. Imipenem discs (10 µg) were placed 20 mm apart from centre to centre on the Mueller Hinton agar plate, and to one of the imipenem discs, 5 µl of 0.5 M EDTA was added. The zone of inhibition of imipenem and imipenem–EDTA discs was compared after 18 hours of incubation at 35 °C. An increase in the zone of inhibition by 7 mm with EDTA when compared to imipenem alone was an indicator of metallo-β-lactamase production (10).

Colistin susceptibility test by Vitek-2

Colistin susceptibility of 17 carbapenem-resistant isolates was tested first by disc diffusion test and then by VITEK 2 Compact. Preparation of inoculum: Aseptically transferred 3 ml of normal saline into clear polystyrene 12x75 mm test tubes. A homogenous suspension of the organism was prepared by transferring 3-4 isolated colonies from the growth to the saline tube and vortexed the suspension for 30 seconds. Adjusted the suspension to the McFarland standard required. The VITEK susceptibility panel was inoculated using this suspension (11).

Detection of Carbapenemase gene by Real-time polymerase chain reaction

Preparation of inoculum for DNA extraction. One colony of *P. aeruginosa* grown on nutrient agar was inoculated into 5 ml Luria Bertani broth and incubated for 2 hours at 37°C with shaking. The broth culture (1.5 ml) was centrifuged at 1800 rpm for 5 minutes. The supernatant was discarded and the deposit was used for DNA extraction (12). The real-time PCR was performed in multiplex PCR, specifically SLAN YGP PCR.

Bacterial DNA extraction

*Pseudomonas D*NA was extracted using a nucleic acid extraction kit (Huwel Life Science India) as per the manufacturer's instructions. The extracted DNA was quantified using a Biophotometer (Eppendorf), and the absorbance was measured at a wavelength of 260 nm. The purity of the DNA was assessed by calculating the ratio of absorbance at 260nm and 280 nm wavelengths**.**

Initial denaturation. The reaction mixture was heated to 95 ℃ for ten minutes to ensure that all double-stranded DNA was fully denatured into single strands. Denaturation: The temperature was maintained at 95°C for 15 seconds to denature the DNA at the beginning of each Polymerase Chain Reaction cycle.

Annealing and extension. Temperature was lowered to 60 °C for 30 seconds, allowing primers to anneal to target DNA and for DNA polymerase to synthesise the new DNA strand. This step was repeated for 40 cycles.

Table 1

The Sequence of the Forward and Reverse Primers Used in the PCR Study (Source-Huwel Life Science, India)

SL. NO	Gene primer	Nucleotides sequence 5'to 3'
1	*bla*$_{VIM}$F	TCT ACA TGA CCG CGT TC
2	*bla*$_{VIM}$R	TGT GCT TTG ACA ACG TTC GC
3	*bla*$_{IMP}$ F	CCA GAT TTA AAA ATA GAG AAG
4	*bla*$_{IMP}$ R	TGG CCA AGC TTC TAC ATT TGC
5	*bla*$_{NDM}$ F	GGT TTT GGC GAT CTG GTT TTC
6	*bla*$_{NDM}$ R	CGG AAT GGC TCA TCA CGA TC
7	*bla*$_{KPC}$ F	GCT ACA CCT AGC TCC ACC
8	*bla*$_{KPC}$ R	ACA GTG GTT AAT CCA TGC
9	*bla*$_{OXA-51}$ F	TAA TGC TTT GAT CGG CCT TG
10	*bla*$_{OXA-51}$ R	TGG ATT GCA CTT CAT CTT GG
11	*bla*$_{OXA-23}$ F	AAA TGA AAC CCC GAG TCA GA
12	*bla*$_{OXA-23}$ R	CCC AAC CAG TCT TTC CAA AA
13	*bla*$_{OXA-48}$ F	TTG GTG GCA TCG ATT ATC GG
14	*bla*$_{OXA-58}$ F	AAG TAT TGG GGC TTG TGC TG
15	*bla*$_{OXA-58}$ R	CCC CTC TGC GCT CTA CAT AC

The plate read: Fluorescent dyes (FAM, HEX, CALFLUOR, Red 610, Cy5, and Cy5.5) were used to monitor the amplification of specific DNA targets in real time. Hold: After the cycling is complete, the reaction was held at 4°C to preserve the amplified DNA.

Ethical considerations

The study was approved by the Institute's ethical committee (KMC/PO/PG thesis/ethics/2019).

Results

Isolates of *P. aeruginosa* strains included in the study were obtained from various clinical samples as follows: (Table 2)**.** Pus sample -52, sputum -19, broncho-alveolar lavage -3, urine-05, catheter sample -11, blood culture -10.

Carbapenemase detection

Carbapenem-resistant 17 isolates of *P. aeruginosa* were subjected to a modified Hodge test and combined disc test. Carbapenemase production was detected in 5 isolates by the modified Hodge test and in 8 isolates by the combined disc test, and PCR detected carbapenemase gene in 8 strains as shown in Table 4.

Detection of carbapenemase genes by Real-Time Polymerase Chain Reaction

A real-time PCR assay of 17 carbapenem-resistant *P. aeruginosa* strains detected 8 MBL producers. In 4 isolates, bla_{NDM} was detected and in 2 isolates bla_{IMP}. Multiple genes, bla_{IMP} and $bla_{NDM,}$ were detected in 1 isolate, and bla_{VIM}, $bla_{IMP,}$ and bla_{NDM} were detected in 1 strain by PCR (Table 5).

Table 2:

Multidrug-resistant P. aeruginosa from different Samples

SL.NO	**Sample type**	**non MDR n=70**	**MDR n=30**
1.	Pus samples	40	12
2.	Sputum samples	15	4
3.	BAL	3	0
4.	Urine	5	11
5.	Blood	7	3
Total	n=100	70	30

Table 3:

Antibiotic Susceptibility Pattern of P. aeruginosa

Antibiotic used	**Antibiotic group as per CLSI**	**Sensitivity n %**	**Resistant n %**
Ceftazidime(30µg)	A	75%	25%
Cefepime (30µg)	B	83%	17%
Aztreonam(30µg)	B	80%	20%
Gentamicin (10 µg)	A	74%	26%
Amikacin (30 µg)	B	84%	16%
Tobramycin (10µg)	A	75%	25%
Ciprofloxacin(5µg)	B	65%	35%
Levofloxacin(5µg)	B	64%	36%
Norfloxacin(10µg)	U	44%	56%
Piperacillin/tazobactam (100/10µg)	B	80%	20%
Imipenem (10µg)	B	83	17%
Meropenem (10µg)	B	84%%	16%
Colistin(10µg)	O	100%	NIL

Discussion

P. aeruginosa is a prominent coloniser and a major cause of infection in almost all sites of the body due to its diverse and complex virulence features. Equally, its ability to survive in adverse environments makes it a tenacious coloniser, predisposing individuals to infections. *P. aeruginosa* is the main cause of morbidity and mortality in cystic fibrosis patients and a nosocomial pathogen which is intrinsically resistant to a wide range of antibiotics.(3,13). It is associated with ventilator-associated pneumonia, central line-associated bloodstream infection, urinary catheter-associated infection, burn and wound infections (4, 14).

P. aeruginosa was recovered from clinical samples: pus (52%), followed by respiratory samples (22%), urinary tract samples (16%), and bloodstream infections (10%). This finding establishes the propensity of these bacteria to cause infections unrestricted to any organs or systems. Studies have shown that Latin America, the Asia Pacific regions, and Europe have the highest rate of *P. aeruginosa* recovery from respiratory samples (15). In the United States, a higher rate of isolation was obtained from the urinary tract, which reflects the increased use of indwelling urinary tract catheters. Various studies from India have reported *P. aeruginosa*, 47.1 % from pus, 36.5% from sputum, 12.5% from urine and 3.8% from blood (16). Another study from India revealed that 54% were from exudates, 32% from urine,6 % from blood, and 8% from sputum samples (17). *Pseudomonas aeruginosa* exhibits intrinsic resistance to many antimicrobial agents through several mechanisms, including biofilm formation, the expression of multiple efflux pumps, low outer membrane permeability, and the production of chromosomally encoded AmpC β-lactamase. In addition, it can acquire resistance genes from other bacteria, leading to the emergence of multidrug-resistant strains (18). However, the frequency of multidrug resistance among our isolates was low.

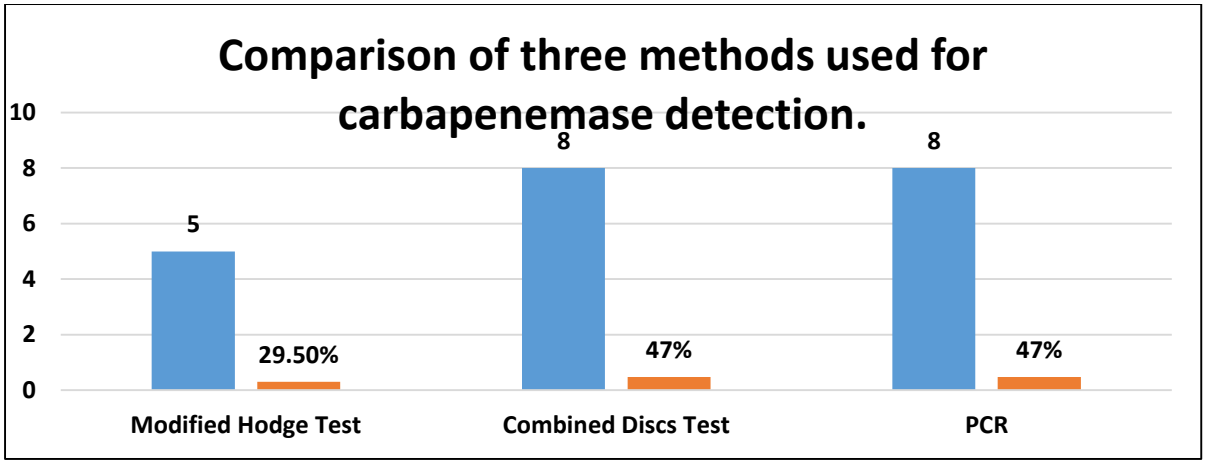

Figure 1
Comparison of Three Methods used for Carbapenemase Detection

Table 4
Carbapenemase Gene Detection by PCR

Target gene	Carbapenem resistant *P. aeruginosa*
bla_{NDM}	4 (23.5%)
bla_{IMP}	2 (11.7 %)
bla_{IMP}&bla_{NDM}	1 (5.8 %)
bla_{VIM}&bla_{IMP}&bla_{NDM}	1 (5.8 %)

In fact, out of 100 isolates of *P. aeruginosa*, 38 strains were pan-susceptible, 32 strains were resistant to one or two classes of antibiotics and 30 strains were resistant to three or more classes of antibiotics. So as per the definition, 30% of the total isolates were multidrug resistant.

The present study revealed that 36% of *P. aeruginosa* strains exhibited resistance against fluoroquinolones such as ciprofloxacin and levofloxacin. A study from Turkey revealed that 12.5% of *P. aeruginosa* showed resistance to ciprofloxacin and 6.9% to levofloxacin (19). The relatively high resistance of 56% found against norfloxacin in the present study may not represent the common resistant pattern, as only 16 out of the 100 isolates were tested from urine.

The activity of beta-lactam antibiotics against *P. aeruginosa* isolates was reasonably high, with ceftazidime and cefepime resistance of 25% and 17 % only. A similar observation was reported by Mario Gajdacs *et al* (20).

Among aminoglycosides tested, *P. aeruginosa* showed a resistance of 26 % to gentamicin, 25% to tobramycin and 16% to amikacin. Resistance to aminoglycosides varies across different geographical areas; for instance, aminoglycoside resistance of 70% (21) and 69.8% (22) was reported in different studies. Conversely, a lower rate of amikacin resistance was observed by Nadeem *et al*, 6.7% (23) and 24 % by Jamshaid *et al* (24), which is in line with the present study. There was a relatively high susceptibility to piperacillin-tazobactam, with only 20 % of the strains being resistant. This result is comparable to 9.6**%** resistance in a study by Nadeem *et al* (23).

Carbapenem resistance

In most circumstances worldwide, carbapenems are the mainstay of treatment against severe infections caused by *P. aeruginosa* (25). Among the carbapenems tested, our isolates showed a resistance of 17% to imipenem and 16% to meropenem, respectively. *P. aeruginosa* showed marginally higher sensitivity to meropenem than imipenem. Studies from India reported a carbapenem resistance of 16% among the *P. aeruginosa* isolates (26), 12% (27), 28.7% (28), 27.6% (29), 42.8% (30), 61% against imipenem and 54% against meropenem, respectively (31). A study from Taiwan observed a lower rate of 10% resistance against carbapenem by *P. aeruginosa* (32). The carbapenem resistance rate found in our study was reasonably lower compared to most of the above studies.

Multidrug resistance in *P. aeruginosa*

The high prevalence of drug resistance leads to limited treatment options and results in treatment failure. Among the MDR *P. aeruginosa*, 30% showed *in-vitro* resistance to 3 or more different antibiotic classes. Similar findings were reported by studies from India at 31.8% (33) and 32.2 %(34), respectively. All carbapenemase-producing strains were sensitive to colistin as per the disc diffusion test and Vitek-2 system. The Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) endorse the broth microdilution test (BMD) for polymyxin and colistin susceptibility (35). However, in our study, we could not perform the same, which is a limitation of the present study.

Limitations of the study

Minimum Inhibitory Concentration (MIC) for the drugs tested was not included in the study.

Conclusions

The study included 100 clinical isolates of *P. aeruginosa*. The majority of the *P. aeruginosa* was obtained from pus samples, followed by isolates from respiratory samples, and urine and blood samples. Out of the 100 samples, 45 samples were from patients in the medical ICU and wards; this was followed in decreasing order by isolates from dermatology, surgical OBG, and ENT departments.

Among the 100 isolates, 30 were MDR *P. aeruginosa*. Maximum resistance was noticed against fluoroquinolones (35%). Out of the 100 isolates, 17 were carbapenem-resistant, of which 9 isolates were from pus samples, 5 from urine and 3 from respiratory samples. In the present study, carbapenemase production

was detected in 5 strains by the modified Hodge test and 8 strains by the combined disc test. Carbapenemase genes were detected by PCR in these 8 isolates. The remaining 9 carbapenem-resistant *P. aeruginosa* isolates were negative for carbapenemase production indicating other mechanisms of carbapenem resistance in these strains. The present study showed 6 *bla* $_{NDM}$ genes in 8 isolates, whereas many previous studies revealed *bla* $_{VIM}$ to be the most prevalent gene in carbapenem-resistant *P. aeruginosa*. All carbapenem-resistant *P. aeruginosa* strains tested were susceptible to colistin, making it the last resort of treatment in such cases.

Recommendations

The study findings highlight the need for antimicrobial stewardship to prevent the spread of carbapenem resistance among *P aeruginosa.*

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Conflict of interest. There is no conflict of interest.

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