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Metallo- β -lactamase production and antimicrobial resistance in *Pseudomonas aeruginosa* from hospital-acquired infections: a comparison of three phenotypic confirmatory tests at a tertiary care centre in Central India

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ABSTRACT

Background: Metallo- β -lactamases (MBLs) are a major cause of carbapenem resistance in *Pseudomonas aeruginosa*, and the prevalence reported by a laboratory depends on the phenotypic test it uses. Institution-specific data are needed to guide empirical therapy.

Objectives: To determine the antimicrobial susceptibility pattern, the prevalence of multidrug resistance (MDR) and the frequency of MBL production among *P. aeruginosa* isolates from hospital-acquired infections, and to compare three phenotypic confirmatory tests for MBL.

Methods: Eighty consecutive, non-duplicate isolates from patients with hospital-acquired infection at a tertiary care hospital in Indore were studied. Susceptibility to nine agents was tested by disc diffusion, and MDR was defined by consensus criteria. All isolates were screened by the modified carbapenem inactivation method (mCIM); screen-positive isolates were tested by the imipenem-EDTA combined disc test (CDT), the double disc synergy test (DDST) and the MBL E-test.

Results: Pus was the commonest specimen (28.75%). Imipenem was the most active agent (90.0% susceptible), and susceptibility was lowest to the ceftazidime-inhibitor combinations (60.0%). Twelve isolates (15.0%; 95% CI 8.8-24.4) were MDR, with significant variation by specimen ($p = 0.038$), from 36.4% in sputum to none in blood. mCIM was positive in 53 isolates (66.3%). The CDT confirmed MBL production in 34, or 42.5% of all isolates (95% CI 32.3-53.4); the DDST and the E-test identified 33 each. MBL frequency did not differ significantly by specimen ($p = 0.635$). Only eight isolates were imipenem-resistant, so at least 26 phenotypic MBL producers were imipenem-susceptible.

Conclusion: Imipenem retained good in-vitro activity and MDR was uncommon. The three EDTA-based tests gave almost identical counts, which supports the inexpensive CDT for routine confirmation. The high phenotypic MBL rate among imipenem-susceptible isolates needs confirmation by carbapenem MICs and PCR before it is used to guide therapy.

Keywords: *Pseudomonas aeruginosa*; metallo- β -lactamase; modified carbapenem inactivation method; combined disc test; multidrug resistance; hospital-acquired infection.

INTRODUCTION

A healthcare-associated infection is defined for surveillance as one that was absent and not incubating at admission and that first meets a site-specific criterion on or after the third hospital day [1]. These infections carry higher rates of antimicrobial resistance than infections acquired in the community. One modelling study estimated the additional hospital cost of antibiotic-resistant infection at a median of about US\$693 billion worldwide in a single year [2]. In the intensive care unit of a North Indian teaching hospital, unit-acquired infection was recorded in 27.9% of patients, and Gram-negative organisms supplied most of the isolates [3].

Pseudomonas aeruginosa is a leading cause of hospital-acquired pneumonia and bacteraemia [4]. Its intrinsic resistance rests on an outer membrane of low permeability, an inducible chromosomal AmpC cephalosporinase and constitutively expressed efflux systems. Acquired β -lactamases, porin loss and target mutations add to this background [4]. The World Health Organization lists carbapenem-resistant *P. aeruginosa* among its priority pathogens [5], and Indian national surveillance continues to report resistance in this organism to agents on which empirical therapy depends [6]. Because resistance rates differ between hospitals within one country [7,8], figures from one centre are a poor guide to prescribing at another.

Metallo- β -lactamases deserve particular attention. They hydrolyse all β -lactams except aztreonam, they are not inhibited by clavulanate or tazobactam, and their genes are often carried on mobile elements [4]. In most Indian diagnostic laboratories they are detected by phenotypic tests. Carbapenemase activity is first screened by the modified carbapenem inactivation method (mCIM), and MBL activity is then confirmed by EDTA-based assays such as the imipenem-EDTA combined disc test (CDT) [9], the double disc synergy test (DDST) and the MBL E-test. These assays do not perform equally. Radhika et al. found that the CDT and the E-test each detected MBL in 75% of carbapenem-resistant isolates, whereas the DDST detected only 41% [10]. Muddassir et al. reported 61.5% by CDT against 81.5% by the modified Hodge test in the same collection [11], and Seyedi et al. found mCIM positivity in 23.7% of isolates but CDT positivity in only 15.4% [12]. EDTA-based detection also depends on the enzyme family: it identified VIM and NDM producers but missed IMP and SPM producers in the evaluation by Gill et al. [13]. When measured against PCR, phenotypic methods may overestimate MBL production [14].

Few Indian studies have applied more than one confirmatory test to a single collection of isolates, so the size of the between-assay difference at a given centre is usually unknown. No such data have been reported from the study institution. The virulence profile of the present collection, examined by phenotypic assays and by PCR for virulence genes, is described in a companion report [15]. This article addresses resistance. Its aims were to determine the antimicrobial susceptibility pattern and the prevalence of MDR among *P. aeruginosa* isolates from hospital-acquired infections, to estimate MBL production by mCIM screening followed by three confirmatory tests, and to describe how MDR and MBL production were distributed across specimen types.

MATERIALS AND METHODS

Study design and setting

This hospital-based, cross-sectional, descriptive study was carried out in the Department of Microbiology, Index Medical College Hospital & Research Centre, Indore, over two years from the date of ethical approval. Specimens were received from the Departments of Medicine, Surgery and Critical Care Medicine and from other clinical departments as required.

Study population

Patients of either sex and any age were eligible when clinical and laboratory evidence of infection first appeared on or after the third day of admission, in line with the CDC/NHSN definition [1]. Eligible specimens were pus and wound exudate, urine, blood, sputum and body fluids yielding *P. aeruginosa*. Only the first isolate per patient per infective episode was included. Isolates from specimens collected within 48 hours of admission were excluded as community-acquired. Isolates judged to represent colonisation, on clinical correlation and, where applicable, quantitative or semi-quantitative culture criteria, were also excluded, as were specimens with inadequate clinical data and isolates from patients who withheld consent.

Sample size

The sample size was calculated with the formula for a single proportion, $n = Z^2p(1 - p)/d^2$, taking $Z = 1.96$, an expected MDR prevalence (p) of 36.5% reported from a comparable Indian tertiary centre [16] and an absolute precision (d) of

10.5%. This gave $n = 80.8$. Eighty consecutive eligible isolates were enrolled, which corresponds to an absolute precision of 10.6%.

Isolation and identification

Specimens were inoculated onto 5% sheep blood agar and MacConkey agar, with cetrimide agar added where mixed or scanty growth was expected. Blood was first incubated in an automated blood culture system and sub cultured when the bottle flagged positive. Plates were incubated aerobically at 35-37°C for 18-24 hours, extended to 48 hours for scanty growth. Presumptive identification was based on colony morphology, pigment production, Gram stain, oxidase and catalase reactions, oxidative metabolism in the Hugh and Leifson test, motility and growth at 42°C. Identity was confirmed on the VITEK 2 Compact system [17]. Confirmed isolates were stored in glycerol at -70°C for batch testing.

Antimicrobial susceptibility testing

Susceptibility was determined by the Kirby-Bauer disc diffusion method on Mueller-Hinton agar with a 0.5 McFarland inoculum, and zone diameters were interpreted by CLSI M100 criteria [18]. The panel comprised amikacin (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), piperacillin (100 µg), carbenicillin (100 µg), ceftazidime (30 µg), ceftazidime-clavulanic acid (30/10 µg), ceftazidime-tazobactam (30/10 µg) and imipenem (10 µg). *P. aeruginosa* ATCC 27853 was used for quality control, and a batch was accepted only when control results fell within the reference ranges.

An isolate was classified as MDR when it was non-susceptible to at least one agent in three or more antimicrobial categories, following Magiorakos et al. [19]. The categories represented in the panel were aminoglycosides, fluoroquinolones, antipseudomonal penicillins, antipseudomonal cephalosporins (with and without a β -lactamase inhibitor) and carbapenems. The extensively drug-resistant and pandrug-resistant categories were not applied, because the panel did not cover the full set of categories those definitions require.

Screening for carbapenemase production

All 80 isolates were screened by the mCIM according to CLSI guidance [18]. A loopful of each isolate was emulsified in tryptic soy broth, a 10 µg meropenem disc was immersed in the suspension and incubated at 35°C for four hours, and the disc was then placed on Mueller-Hinton agar seeded with *Escherichia coli* ATCC 25922. After overnight incubation, a zone of 6-15 mm, or pinpoint colonies within a zone of 16-18 mm, was read as positive, and a zone of 19 mm or more as negative.

Phenotypic confirmation of MBL production

Isolates positive on mCIM were tested by three EDTA-based methods. In the CDT, zones around an imipenem (10 µg) disc and an imipenem disc supplemented with EDTA were compared on the same plate, and an increase of 7 mm or more with EDTA was read as positive [9]. In the DDST, an imipenem (10 µg) disc and an EDTA disc were placed apart, centre to centre, and enhancement of the imipenem zone towards the EDTA disc was read as positive [10]. The MBL E-test strip, carrying an imipenem gradient at one end and imipenem with EDTA at the other, was read as positive when the ratio of imipenem MIC to imipenem-EDTA MIC was 8 or more, or when a phantom zone or deformation of the ellipse was seen [10]. MBL-positive and MBL-negative reference strains were included in each run.

For prevalence estimates, an isolate was classed as an MBL producer when it was positive on the CDT. Isolates negative on mCIM were regarded as non-producers and were not tested further.

Statistical analysis

Data were analysed in jamovi. Categorical variables were summarised as frequencies and percentages, and 95% confidence intervals for proportions were calculated by the Wilson score method. The distributions of MDR and of MBL production across the five specimen groups were compared by the chi-square test. Because several expected cell counts were below five, these p values were checked by a Monte Carlo permutation test with 200,000 replicates. A p value below 0.05 was taken as significant.

RESULTS

Demographic and specimen profile

Of the 80 isolates, 50 (62.5%) came from male and 30 (37.5%) from female patients, a ratio of 1.67:1. Patients aged 30 years or below contributed 41 isolates (51.3%), and the 10-20 year group was the largest single group (23; 28.8%). Pus was the commonest specimen (23; 28.75%), followed by urine (17; 21.25%), blood (16; 20.0%), body fluid (13; 16.25%) and sputum (11; 13.75%) (Table 1).

Among pus isolates, diabetic foot ulcer was the commonest source (7/23; 30.4%), followed by chronic suppurative otitis media (4; 17.4%). Six of the 17 urine isolates (35.3%) came from catheter-associated urinary tract infection. All sputum isolates came from respiratory tract infection, and blood isolates came from patients with fever (10; 62.5%) or pneumonia (6; 37.5%). The body fluid isolates were recovered from patients with chronic obstructive pulmonary disease (COPD), ischaemic heart disease (IHD), or both.

Table 1. Demographic and specimen profile of the *P. aeruginosa* isolates (n = 80)

Characteristic	Category	n (%)
Sex	Male	50 (62.5)
	Female	30 (37.5)
Age group (years)	10-20	23 (28.8)
	21-30	18 (22.5)
	31-50	14 (17.5)
	51-60	14 (17.5)
	61-70	11 (13.8)
Specimen	Pus	23 (28.8)
	Urine	17 (21.3)
	Blood	16 (20.0)
	Body fluid	13 (16.3)
	Sputum	11 (13.8)
Leading clinical condition within specimen group	Pus: diabetic foot ulcer	7/23 (30.4)
	Urine: urinary tract infection / catheter-associated UTI	11/17 (64.7) / 6/17 (35.3)
	Sputum: respiratory tract infection	11/11 (100)
	Blood: fever / pneumonia	10/16 (62.5) / 6/16 (37.5)
	Body fluid: COPD / IHD with COPD / IHD	5/13 (38.5) / 5/13 (38.5) / 3/13 (23.1)

UTI, urinary tract infection; COPD, chronic obstructive pulmonary disease; IHD, ischaemic heart disease.

Antimicrobial susceptibility and multidrug resistance

Imipenem was the most active agent, with 72 isolates (90.0%) susceptible and 8 (10.0%; 95% CI 5.2-18.5) resistant. Amikacin and gentamicin were each active against 59 isolates (73.75%), and ciprofloxacin against 56 (70.0%). Ceftazidime and piperacillin were each active against 53 (66.25%) and carbenicillin against 51 (63.75%). Susceptibility was lowest to ceftazidime-clavulanic acid and ceftazidime-tazobactam, at 48 isolates (60.0%) each (Table 2).

Table 2. Antimicrobial susceptibility of *P. aeruginosa* isolates (n = 80)

Antimicrobial agent (disc content)	Susceptible n (%)	Resistant n (%)	Resistance, 95% CI (%)
Imipenem (10 µg)	72 (90.00)	8 (10.00)	5.2-18.5
Amikacin (30 µg)	59 (73.75)	21 (26.25)	17.9-36.8
Gentamicin (10 µg)	59 (73.75)	21 (26.25)	17.9-36.8
Ciprofloxacin (5 µg)	56 (70.00)	24 (30.00)	21.1-40.8
Ceftazidime (30 µg)	53 (66.25)	27 (33.75)	24.3-44.6

Antimicrobial agent (disc content)	Susceptible n (%)	Resistant n (%)	Resistance, 95% CI (%)
Piperacillin (100 µg)	53 (66.25)	27 (33.75)	24.3-44.6
Carbenicillin (100 µg)	51 (63.75)	29 (36.25)	26.6-47.2
Ceftazidime-clavulanic acid (30/10 µg)	48 (60.00)	32 (40.00)	30.0-51.0
Ceftazidime-tazobactam (30/10 µg)	48 (60.00)	32 (40.00)	30.0-51.0

CI, confidence interval (Wilson score method).

Twelve isolates (15.0%; 95% CI 8.8-24.4) were MDR. The proportion differed significantly between specimen groups ($\chi^2 = 10.15$, $df = 4$, $p = 0.038$; Monte Carlo $p = 0.035$). MDR was commonest among sputum (4/11; 36.4%) and body fluid (4/13; 30.8%) isolates, uncommon among urine (2/17; 11.8%) and pus (2/23; 8.7%) isolates, and absent among blood isolates (Table 3).

Table 3. Distribution of multidrug-resistant *P. aeruginosa* by specimen type

Specimen (n)	MDR n (%)	Non-MDR n (%)
Pus (23)	2 (8.7)	21 (91.3)
Urine (17)	2 (11.8)	15 (88.2)
Sputum (11)	4 (36.4)	7 (63.6)
Blood (16)	0 (0.0)	16 (100.0)
Body fluid (13)	4 (30.8)	9 (69.2)
Total (80)	12 (15.0)	68 (85.0)

MDR, multidrug-resistant. $\chi^2 = 10.15$, $df = 4$, $p = 0.038$ (Monte Carlo permutation $p = 0.035$).

Carbapenemase screening and MBL confirmation

The mCIM was positive in 53 isolates (66.3%; 95% CI 55.4-75.7) and negative in 27 (33.8%). Of the 53 screen-positive isolates, the CDT identified 34 (64.2%) as MBL producers, and the DDST and the MBL E-test each identified 33 (62.3%) (Table 4). On the CDT definition, 34 of the 80 isolates were MBL producers, a prevalence of 42.5% (95% CI 32.3-53.4). Only 8 isolates were resistant to imipenem on disc diffusion. At least 45 of the 53 mCIM-positive isolates, and at least 26 of the 34 CDT-positive isolates, were therefore imipenem-susceptible by disc diffusion.

Table 4. Carbapenemase screening and phenotypic confirmation of MBL production

Test	Isolates tested	Positive n	% of tested (95% CI)	% of all isolates (95% CI)
mCIM (screening)	80	53	66.3 (55.4-75.7)	66.3 (55.4-75.7)
Imipenem-EDTA combined disc test	53	34	64.2 (50.7-75.7)	42.5 (32.3-53.4)
Double disc synergy test	53	33	62.3 (48.8-74.1)	41.3 (31.1-52.2)
MBL E-test	53	33	62.3 (48.8-74.1)	41.3 (31.1-52.2)

mCIM, modified carbapenem inactivation method; MBL, metallo- β -lactamase; CI, confidence interval (Wilson score method). Confirmatory tests were performed on mCIM-positive isolates only.

MBL production was numerically most frequent among blood isolates (8/16; 50.0%), followed by pus (11/23; 47.8%) and urine (8/17; 47.1%), and least frequent among body fluid (4/13; 30.8%) and sputum (3/11; 27.3%) isolates. The difference between specimen groups was not significant ($\chi^2 = 2.56$, $df = 4$, $p = 0.635$; Monte Carlo $p = 0.654$) (Table 5).

Table 5. Distribution of MBL producers by specimen type (n = 80)

Specimen (n)	MBL producers n (%)	Non-producers n (%)
Pus (23)	11 (47.8)	12 (52.2)
Urine (17)	8 (47.1)	9 (52.9)
Blood (16)	8 (50.0)	8 (50.0)
Body fluid (13)	4 (30.8)	9 (69.2)
Sputum (11)	3 (27.3)	8 (72.7)
Total (80)	34 (42.5)	46 (57.5)

MBL production defined by a positive imipenem-EDTA combined disc test. $\chi^2 = 2.56$, $df = 4$, $p = 0.635$ (Monte Carlo permutation $p = 0.654$).

DISCUSSION

This study describes the resistance profile of 80 *P. aeruginosa* isolates from hospital-acquired infections at a tertiary care centre in Central India. Imipenem remained the most active agent and MDR was uncommon. By contrast, carbapenemase activity was detected in two-thirds of isolates, and EDTA-based tests classed 42.5% as MBL producers, most of which tested imipenem-susceptible. The three confirmatory tests gave almost identical counts.

Susceptibility rates here were higher than at most comparable centres. In a five-year study from intensive care units in eastern India, only 56% of isolates were susceptible to amikacin, and resistance to imipenem reached 51% [20]. In Varanasi, meropenem was active against 68% of isolates [21]. A meta-analysis of Ethiopian healthcare-associated isolates found the lowest pooled resistance to amikacin (20.9%) and meropenem (28.64%) [22]. An imipenem susceptibility of 90% therefore lies at the favourable end of the published range.

The MDR prevalence of 15.0% was also lower than that reported from Lucknow (36.5%) [16], Iran (31.42%) [23] and Nepal (50%) [24], and far below the figures above 80% reported by Alharbi et al. [25] and in the Ethiopian meta-analysis [22]. Part of this difference may be real. Declines in resistance have been documented after antimicrobial stewardship and infection control were strengthened [8,20]. Part of it is probably methodological. The panel of nine discs represented five categories and omitted several agents that other studies test, including cefepime, piperacillin-tazobactam, aztreonam and the polymyxins. An isolate has fewer chances to cross the three-category threshold on a narrow panel, so our MDR figure should be read as a conservative estimate.

MDR was concentrated in sputum and body fluid isolates and was not found in any blood isolate, and this difference remained significant on exact testing. Sputum isolates came from respiratory tract infection, and body fluid isolates from patients with COPD or IHD. Such patients often have longer admissions and more antibiotic exposure, although these variables were not recorded here. Carbapenem resistance in 58.6% of Gram-negative isolates from ventilator-associated pneumonia in Lucknow [26] points in the same direction. The subgroups were small, and the finding needs confirmation in a larger series. If it holds, empirical antipseudomonal policy at this centre could reasonably differ by site of infection. The phenotypic MBL prevalence of 42.5% is close to the 40.84% reported from Peshawar [27] and the 48% reported among imipenem-resistant burn isolates in Iran [28]. It is lower than the 66.7% among MDR isolates in Kathmandu [24] and the 56% among carbapenem-resistant environmental isolates in Dhaka [29]. These comparisons need care. Most studies apply confirmatory tests only to carbapenem-resistant isolates, whereas in the present study every mCIM-positive isolate was tested, whatever its imipenem result. The denominators therefore differ, and prevalence among all isolates cannot be compared directly with prevalence among resistant isolates.

Agreement between the three confirmatory tests was close: 34 isolates by CDT and 33 each by DDST and E-test. This contrasts with the study of Radhika et al., in which the DDST detected only 41% of MBL producers against 75% for the other two methods [10]. Differences in disc spacing, EDTA concentration and reading criteria affect DDST sensitivity, and the test may have been performed under favourable conditions here. It is also possible that MBL activity in this collection was strongly expressed. These counts are aggregates, however, and agreement at the level of the individual isolate was not analysed. Two tests can give the same total while disagreeing on particular isolates. Even so, the similar totals support the CDT as a practical routine method at this centre, since it is cheaper than the E-test and easier to read than the DDST. Radhika et al. reached a similar recommendation [10].

The most important finding needs to be stated plainly. Fifty-three isolates were mCIM-positive and 34 were confirmed as MBL producers, yet only eight were imipenem-resistant on disc diffusion. A collection in which more than 40% of isolates produce an MBL would be expected to show much higher carbapenem resistance. Several explanations are possible. MBL expression may be low or inducible, and an MBL alone may not raise the imipenem MIC above the breakpoint unless porin loss or efflux is also present. Test conditions matter as well: zinc content of the medium influences whether MBL-mediated resistance is detected [30]. False-positive results are a further possibility. EDTA has intrinsic activity against *P. aeruginosa* and increases outer-membrane permeability, so it can enlarge zones without any MBL, and phenotypic methods have overestimated MBL production against PCR in other collections [14]. Discordance between phenotype and genotype is a recognised source of error in carbapenemase detection [31]. A recording error in the screening or susceptibility data cannot be excluded either. Carbapenem MICs on the mCIM-positive isolates, and PCR for blaNDM, blaVIM and blaIMP, would resolve the question. Until then, the MBL prevalence reported here should be treated as provisional.

Screening and confirmation also measure different things. The mCIM detects carbapenemase activity of any class. The 19 isolates that were mCIM-positive but CDT-negative may carry serine carbapenemases, or may be false-positive screens. The EDTA-modified CIM can separate these groups; CLSI describes it for Enterobacterales, and Gill et al. have evaluated it in *P. aeruginosa* [13]. Ahmad et al. used it in Lucknow and found blaNDM in every phenotypically positive isolate, with blaOXA-48 in 85% [16]. Adding the eCIM, or a stepwise algorithm of the kind proposed by Mancini et al. [32], would sharpen the classification at this centre.

MBL production was numerically highest among blood isolates, but the difference between specimens was not significant. The data therefore do not show enrichment of MBL producers in bloodstream infection. Carbapenemase screening of blood isolates remains sensible on clinical grounds, because bacteraemia carries high mortality and its treatment depends heavily on carbapenems.

Strengths and limitations

Every screen-positive isolate was tested by three confirmatory methods, which allowed their yields to be compared within one collection. Isolates were consecutive, non-duplicate and drawn from five specimen types, and the definition of hospital-acquired infection was applied prospectively.

The study also has limitations. The sample was modest, and subgroup estimates, particularly for the 12 MDR isolates, are imprecise. The antimicrobial panel was narrow, MICs were not determined, and colistin was not tested. No molecular confirmation of MBL genes was performed, which leaves the discordance between MBL phenotype and imipenem susceptibility unresolved. mCIM-negative isolates were not tested by the confirmatory methods, and isolate-level agreement between the three tests was not analysed. Data on prior antibiotic exposure, device use and outcome were not collected, so the specimen-related pattern of MDR could not be explained. Finally, the findings describe a single centre and should not be extrapolated to other institutions.

CONCLUSION

Imipenem retained good in-vitro activity against *P. aeruginosa* from hospital-acquired infections at this centre, and MDR was uncommon, although it was concentrated in respiratory and body fluid isolates. Three EDTA-based tests identified almost the same number of MBL producers, which supports the CDT for routine confirmation. The phenotypic MBL rate of 42.5%, largely among imipenem-susceptible isolates, is not consistent with the low carbapenem resistance observed. It should be verified by carbapenem MICs and PCR before it is used to guide empirical therapy. Routine carbapenemase screening, especially of bloodstream isolates, is recommended.

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