



METALLO-B-LACTAMASE PHENOTYPIC AND MOLECULAR DETECTION IN IMIPENEM-RESISTANT GRAM-NEGATIVE BACILLI FROM A TERTIARY CARE HOSPITAL IN CENTRAL INDIA

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Abstract -

Background: The ability of metallo- β -lactamase (MBL)-producing Gram-negative bacilli to hydrolyze carbapenems, which are frequently employed as last-resort antibiotics, poses a significant therapeutic problem. In India's tertiary care facilities, where carbapenem resistance is rising, particularly among non-fermenters, this issue is very serious.

AIM- In a tertiary care hospital in Central India, the current study sought to ascertain the frequency of carbapenem-resistant Gram-negative bacilli and to assess phenotypic and genetic techniques for MBL production detection.

Materials and Methods: The Department of Microbiology at the Index Medical College Hospital and Research Center in Indore carried out a cross-sectional observational study. Standard microbiological methods were used to process 900 clinical samples. Conventional biochemical techniques were used to identify isolates that were gram-negative. The Kirby-Bauer method was used to test for antimicrobial susceptibility. The Modified Hodge Test (MHT) and EDTA Disc Synergy (EDS) were used to screen isolates that were resistant to carbapenem for the generation of MBL. PCR was used to detect the blaIMP gene molecularly.

Results: Gram-negative bacteria were found in 524 (58.2%) of the 900 samples; 231 (25.7%) of them were resistant to carbapenem. The most common resistant species were *Acinetobacter baumannii* (48.0%) and *Klebsiella pneumoniae* (38.6%). EDS and MHT positivity rates for phenotypic MBL identification were 28.1% and 24.2%, respectively. 7.2% of carbapenem-resistant isolates had the blaIMP gene identified by PCR. Male patients and older individuals showed greater resistance.

Conclusion: In this context, carbapenem resistance and MBL generation are serious issues. While molecular techniques offer supporting data, phenotypic methods are still useful for routine screening. Effective surveillance, infection control, and antimicrobial stewardship need the combined application of both strategies.

Keywords –MBL, PCR, EDS, MHT

Introduction -

The emergence of metallo-β-lactamase (MBL)-producing Gram-negative bacilli poses a significant threat to antimicrobial therapy, particularly due to their ability to hydrolyse carbapenems like imipenem, which are last-resort drugs for multidrug-resistant infections. ¹ In tertiary care hospitals, especially in Central India, imipenem-resistant isolates from clinical samples such as urine, sputum, and blood have shown alarming prevalence rates of MBL production, often exceeding 80% among resistant strains. ² These enzymes, encoded by genes like blaNDM-1, confer resistance through zinc-dependent hydrolysis, complicating treatment in intensive care units where nosocomial infections predominate. ³ Phenotypic detection methods, including the combined disc synergy test (CDST), double-disc synergy test (DDST), modified Hodge test (MHT), and E-test, offer rapid screening by observing enhanced inhibition zones with EDTA, a metal chelator. ⁴ Molecular techniques, such as PCR targeting MBL genes, provide confirmatory evidence but are less accessible in resource-limited settings. ⁵ Studies from Indian hospitals report MBL positivity in 18-91% of imipenem-resistant *Pseudomonas aeruginosa* and *Acinetobacter spp.* underscoring the need for routine surveillance. ⁶ In Central India, similar trends highlight non-fermenters as primary culprits, with Enterobacteriaceae also implicated. Accurate detection guides infection control and antibiotic stewardship, preventing outbreaks. This study evaluates both phenotypic and molecular approaches in a Central India tertiary care hospital to determine MBL prevalence and optimize diagnostics. Early identification is crucial amid rising carbapenem resistance in Gram-negative bacilli. ⁷

Material and method –

Study site -The present study was conducted at the Department of Microbiology, Index Medical College Hospital and Research Centre, Indore (M.P).

Study design- The present study is **cross-sectional observational study**.

Study duration – The study duration of 18th month including 6 months of data analysis.

Ethical clearance- Ethical clearance was obtained from the institutional ethical committee. Ethical clearance letter has been enclosed in this thesis.

Inclusion Criteria

- Patients diagnosed with urinary tract infections.
- Patients presenting with respiratory tract infections and prolonged fever (>3–5 days).
- Patients with diabetes mellitus, including those with diabetic foot ulcers.
- Patients with burns or wound infections.
- Patients who have undergone surgical procedures and developed post-operative infections.
- Patients with indwelling medical devices or catheters.

Exclusion Criteria

- Patients with infections caused exclusively by Gram-positive bacteria.
- Patients who refused to provide informed consent for participation.
- Clinical specimens that were inadequate, contaminated, or unsuitable for microbiological processing.

Sample size -The study was conducted in the bacteriology section of the microbiology lab at the Index Medical College Hospital and Research Center in Indore, Madhya Pradesh. The sample size was calculated using the following expression:

$$n = Z^2 \times P \times (1 - P) / d^2$$

Z = statistics for a level of confidence

P = Expected prevalence or proportions

Considering prevalence of MBL-producing Gram-negative bacilli in Central India $\approx 30\%$ ($p=0.30$), confidence level 95% ($Z=1.96$) and precision 5% ($d=0.05$), the calculated sample size was:

$$n = (1.96)^2 \times 0.30 \times 0.70 / (0.05)^2 \approx 900$$

Specimen collection –

Clinical samples were cultured on Nutrient agar, Blood agar, MacConkey agar, and Chocolate agar. Selective media were used as required.

- **Blood:** Inoculated into BHI broth, incubated for 18–24 hours, then subculture.
- **Urine:** Examined by wet mount and cultured on CLED, Blood, Nutrient, MacConkey, and Chocolate agar.
- **Pus/Sputum:** Gram-stained and cultured on routine media.
- **Stool:** Enriched in Selenite F broth and subculture on MacConkey, DCA, and TCBS agar.

Culture Media

Clinical specimens were inoculated onto Blood agar, MacConkey agar, Chocolate agar, Selenite F broth, Xylose Lysine Deoxycholate (XLD) agar, Deoxycholate Citrate Agar (DCA), and Nutrient agar. Gram-negative bacilli were isolated and differentiated based on hemolytic patterns, lactose fermentation, and selective or differential inhibitory properties of the media.

Identification Methods

Isolates were subjected to Gram staining, motility testing, and standard biochemical reactions including catalase, oxidase, Triple Sugar Iron (TSI) agar, IMViC tests, and urease test. These methods enabled identification of *Escherichia coli*, *Klebsiella spp.*, *Proteus spp.*, *Salmonella spp.*, *Shigella spp.*, *Pseudomonas spp.*, and *Citrobacter spp.*

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed using the Kirby–Bauer disc diffusion method on Mueller–Hinton agar. Minimum inhibitory concentrations (MICs) were determined by the agar dilution method to assess resistance to imipenem.

Phenotypic Detection of Metallo- β -Lactamase (MBL)

Phenotypic detection of MBL production in imipenem-resistant isolates was carried out using the EDTA disc synergy test, Modified Hodge Test, and MIC reduction assay with EDTA, confirming metallo- β -lactamase activity.

Molecular Detection of MBL Genes

Genomic and plasmid DNA were extracted from confirmed isolates. Polymerase chain reaction (PCR) was performed for detection of the *bla_{IMP}* gene using specific primers (P1 and P2). PCR products were analyzed by agarose gel electrophoresis.

Result –

A total of 900 clinical samples were processed, of which 524 (58.2%) yielded Gram-negative bacilli. *Escherichia coli* was the most common isolate (18.3%), followed by *Klebsiella* species (14.4%). Overall, 231 isolates (25.7%) were carbapenem-resistant. Resistance was highest to imipenem (25.7%) and meropenem (24.3%). *Acinetobacter baumannii* was the predominant carbapenem-resistant organism (48.0%), followed by *Klebsiella pneumoniae* (38.6%). Carbapenem resistance was more common in males (64.3%) and in the 61–80 year age group (40.3%).

The sample-wise distribution of carbapenem-resistant Gram-negative isolates showed that *Acinetobacter baumannii* was most frequently isolated from blood and respiratory samples, with 35 and 34 isolates, respectively, followed by pus/wound samples (21), urine (17), and stool (4), totaling

111 isolates. *Klebsiella pneumoniae* was predominantly recovered from urine samples (33), followed by blood (20), pus/wound (16), respiratory samples (16), and stool (4), amounting to 89 isolates. *Pseudomonas aeruginosa* was detected in urine (4), pus/wound (4), blood (3), respiratory samples (3), and stool (1), totaling 15 isolates. *Escherichia coli* was isolated from urine (6), pus/wound (2), blood (1), and stool (1), summing to 10 isolates. *Enterobacter* species were recovered from blood (2), urine (1), and pus/wound (1), totaling 4 isolates, while *Citrobacter* species were isolated from urine (1) and pus/wound (1), totaling 2 isolates. Overall, 231 carbapenem-resistant isolates were identified across all sample types.

Table 1- Sample-wise Distribution of Carbapenem-Resistant Isolates

Bacterial species	Urine	Blood	Pus/Wound	Respiratory	Stool	Total
<i>Acinetobacter baumannii</i>	17	35	21	34	4	111
<i>Klebsiella pneumoniae</i>	33	20	16	16	4	89
<i>Pseudomonas aeruginosa</i>	4	3	4	3	1	15
<i>Escherichia coli</i>	6	1	2	0	1	10
<i>Enterobacter</i> species	1	2	1	0	0	4
<i>Citrobacter</i> species	1	0	1	0	0	2
Total	62	61	45	53	10	231

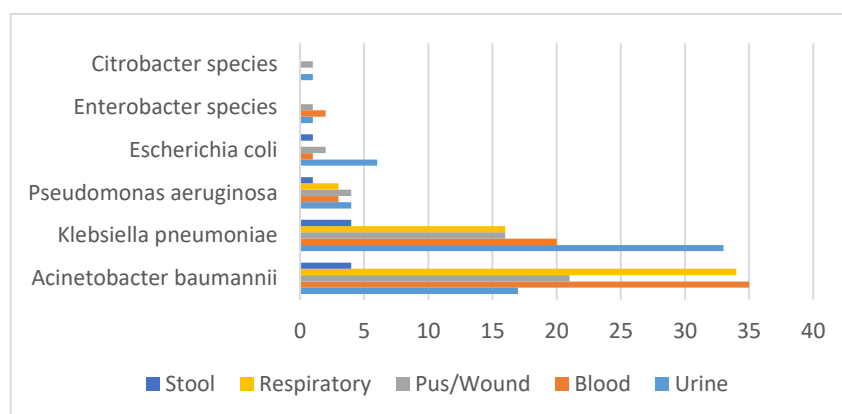


Figure 1- Sample-wise Distribution of Carbapenem-Resistant Isolates

Phenotypic detection of metallo- β -lactamase (MBL) among carbapenem-resistant Gram-negative isolates was performed using EDTA Disc Synergy (EDS) and Modified Hodge Test (MHT). Among 111 *Acinetobacter baumannii* isolates tested, 26 (23.4%) were EDS positive and 24 (21.6%) were MHT positive. For *Klebsiella pneumoniae*, 22 out of 89 isolates (24.7%) were EDS positive, while 20 isolates (22.5%) were MHT positive. In *Pseudomonas aeruginosa*, 7 of 15 isolates (46.7%) showed EDS positivity and 6 isolates (40.0%) were MHT positive. Among 10 *Escherichia coli* isolates, 4 (40.0%) were EDS positive and 3 (30.0%) were MHT positive. For *Enterobacter* species, 2 out of 4 isolates (50.0%) were positive by both EDS and MHT. Both *Citrobacter* species tested (2 isolates) were EDS positive, while 1 isolate (50.0%) was MHT positive. Overall, out of 231 carbapenem-resistant isolates, 65 (28.1%) were EDS positive and 56 (24.2%) were MHT positive.

Table 2- Phenotypic Detection of MBL by EDS and MHT

Bacterial species	Total tested	EDS positive n (%)	MHT positive n (%)
<i>Acinetobacter baumannii</i>	111	26 (23.4)	24 (21.6)
<i>Klebsiella pneumoniae</i>	89	22 (24.7)	20 (22.5)
<i>Pseudomonas aeruginosa</i>	15	7 (46.7)	6 (40.0)
<i>Escherichia coli</i>	10	4 (40.0)	3 (30.0)
<i>Enterobacter</i> species	4	2 (50.0)	2 (50.0)

<i>Citrobacter</i> species	2	2 (100)	1 (50.0)
Total	231	65 (28.1%)	56 (24.2%)

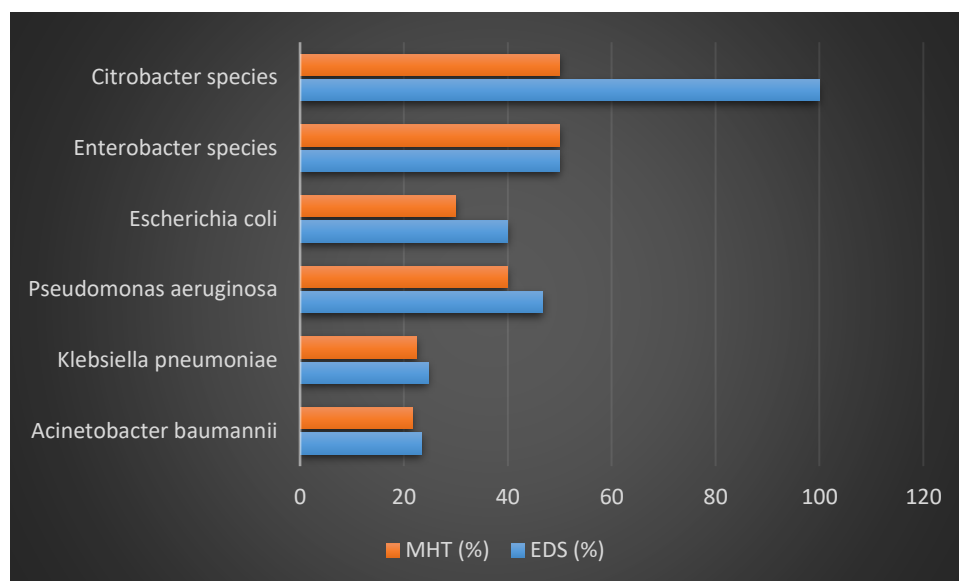


Figure 2-Phenotypic Detection of MBL by EDS and MHT

Table 3- Comparison of Phenotypic and Genotypic Methods

Detection method	MBL-positive isolates (n)	Percentage (%)
EDTA Disc Synergy (EDS)	65	28.1%
Modified Hodge Test (MHT)	56	24.2 %
PCR (blaIMP gene)	17	7.2 %

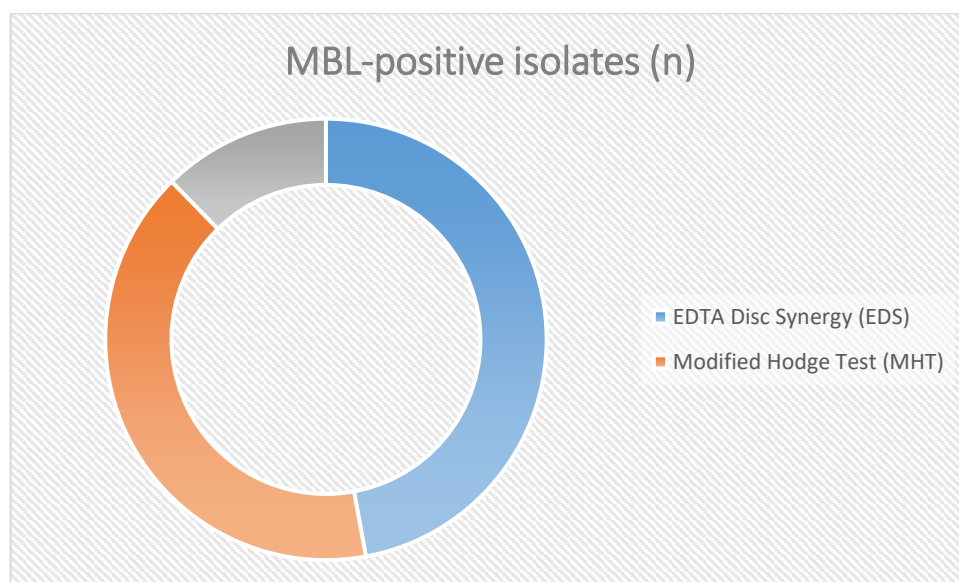


Figure 1-Comparison of Phenotypic and Genotypic Methods

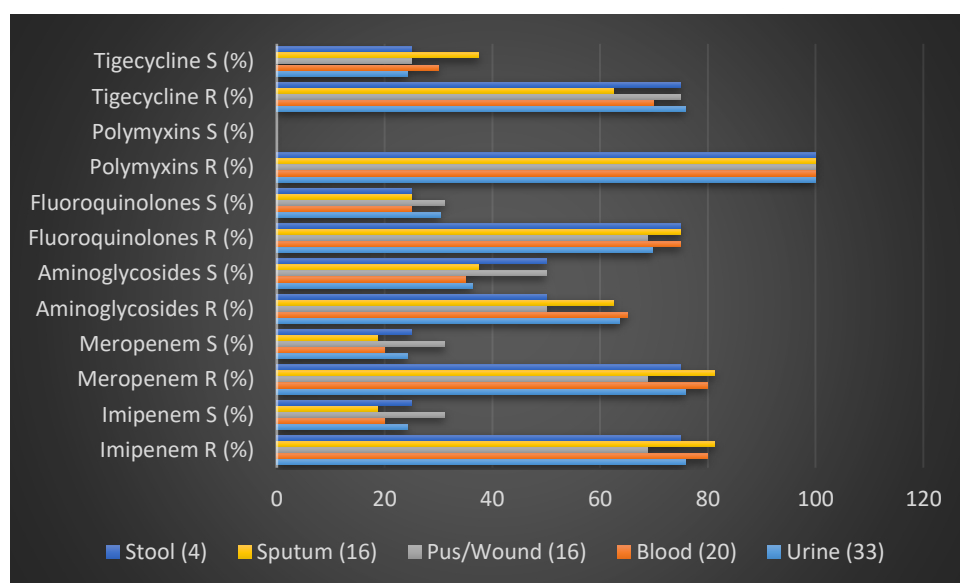


Figure 4- Specimen-wise Antibiotic Resistance and Susceptibility Pattern of Carbapenem-Resistant *Klebsiella pneumoniae*

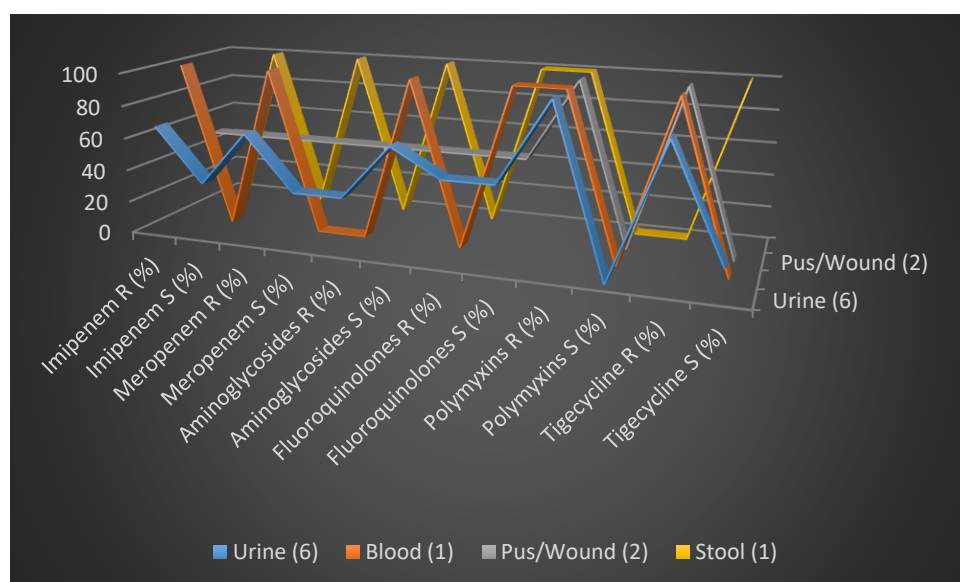


Figure 5- Specimen-wise Antibiotic Resistance and Susceptibility Pattern of Carbapenem-Resistant *Escherichia coli* (n = 10)

Discussion

This study found a **carbapenem resistance rate of 25.7%** among 900 clinical samples yielding Gram-negative bacilli, with *Acinetobacter baumannii* (48.0%) and *Klebsiella pneumoniae* (38.6%) as the main resistant organisms. These rates are similar to several Indian reports but higher than some. For example, Gupta et al. (2022)⁸ in Bihar reported 16.8% carbapenem-resistant Gram-negative bacteria (CR-GNB), Wattal et al. (2017)⁹ in Delhi observed 13–51% in tertiary hospitals, and Nair et al. (2021)¹⁰ reported 12.3% in Mumbai. Regional ICU data from the Indian Antimicrobial Resistance Surveillance Network (AMRSN 2021)¹¹ show even higher rates, with >70% for *A. baumannii* and >50% for *K. pneumoniae*. *A. baumannii* dominated carbapenem-resistant isolates (111/231), mainly from blood (35 isolates) and respiratory samples (34), followed by *K. pneumoniae* (89 isolates), most commonly from urine (33). This pattern matches ICU trends, with *A. baumannii* resistance reported at 87.5% in AMRSN data and in bacteremia studies by Singh et al. (2023).¹² Phenotypic metallo- β -lactamase (MBL) detection showed 28.1% positivity with EDTA Disc Synergy (EDS) and 24.2% with Modified Hodge Test (MHT), highest in *Pseudomonas aeruginosa* (46.7% EDS) and

Enterobacter species (50%). Genotypic PCR for blaIMP was lower at 7.2%. These results are similar to Panchal et al. (2017),¹³ who reported high MBL rates (91.5%) and noted that EDS was more sensitive than other disk tests. Resistance patterns showed high rates for imipenem (25.7% overall, 75–82% in major species) and meropenem (24.3%), along with fluoroquinolone resistance (up to 88%) and tigecycline resistance (70–83%). Polymyxins remained fully effective. Tigecycline resistance in 7.85% of dual-resistant Gram-negative isolates aligns with Kumar et al. (2024),¹⁴ supporting the role of polymyxins as a last-resort option, consistent with CRAB/CRKP studies by Sharma et al. (2022).¹⁵ Demographic analysis showed higher resistance in males (64.3%) and the 61–80-year age group (40.3%), likely linked to hospitalization and comorbidities, as noted by Gupta et al. (2022).¹⁶

Phenotypic detection of MBL using EDTA Disc Synergy (EDS) and Modified Hodge Test (MHT) revealed positivity rates of 28.1% and 24.2%, respectively. These findings are comparable with those of Yong et al. (2009),⁷ who demonstrated higher sensitivity of EDTA-based methods for detecting MBL production. Franklin et al. (2006)¹⁷ reported that MHT, although useful, lacks specificity and may detect non-MBL carbapenemases, which explains the lower positivity rate observed in the present study.

PCR-based detection revealed MBL genes in 7.2% of carbapenem-resistant isolates, with *Acinetobacter baumannii* showing the highest gene positivity. Similar results were reported by Kumarasamy et al. (2010)¹⁸ and Walsh et al. (2011)¹⁹ who observed lower genotypic positivity compared to phenotypic methods. The discrepancy between phenotypic and genotypic detection may be due to the presence of other resistance mechanisms, gene variants not included in the PCR panel, or overestimation by phenotypic tests.

The higher detection rate of phenotypic methods compared to PCR highlights the importance of using a combination of diagnostic approaches. Queenan and Bush (2007)²⁰ emphasized that phenotypic methods are suitable for routine screening in diagnostic laboratories, whereas molecular methods serve as confirmatory tools. The findings of the present study support this recommendation.

Conclusion –

The current investigation shows a substantial burden of carbapenem-resistant Gram-negative bacteria in a tertiary care hospital setting, with non-fermenters emerging as important pathogens. Metallo- β -lactamase was often produced by carbapenem-resistant isolates, particularly in species associated with serious and invasive diseases. Molecular assays provided validation of resistance mechanisms, while phenotypic approaches were useful for routine screening. Early detection must be combined with effective infection control and antimicrobial stewardship to prevent the spread of these highly resistant bacteria.

Reference -

1. Sachdeva R, et al. Evaluation of different phenotypic tests for detection of metallo-lactamases in imipenem resistant *Pseudomonas aeruginosa*. **J Lab Physicians**. 2016;8(2):102–106.
2. Prevalence of metallo-beta-lactamases (MBLs) producing *Pseudomonas* spp. and *Acinetobacter* spp. in a tertiary care hospital in India. **Int J Res Med Sci**. 2018;6(4):5255.
3. Phenotypic Methods for the Detection of Metallo-Beta-Lactamase Production by Gram-Negative Bacteria. **J Microbiol Immunol**. 2021.
4. Metallo-Beta-Lactamase Production in *Pseudomonas aeruginosa* Causing Hospital-Acquired Infections. **Healthcare Bulletin**. 2025.
5. Gupta V. Metallo- β -lactamase producing nonfermentative gram-negative bacilli. **J Microbiol Immunol Infect**. 2012.
6. Phenotypic identification and molecular detection of blaNDM-1 gene in multidrug-resistant Gram-negative bacilli. **Indian J Med Res**. 2025.
7. Deshmukh DG, et al. Metallo- β -lactamase-producing clinical isolates from patients of a tertiary care hospital. **J Lab Physicians**. 2011;3(2):93–97.

8. Gupta V, et al. Carbapenem-resistant Gram-negative bacteria in Bihar, India. *J Clin Diagn Res.* 2022;16(3):DC01–DC06.
9. Wattal C, Goel N, Oberoi JK. Carbapenem resistance among Gram-negative bacteria in Delhi tertiary hospitals. *Indian J Med Microbiol.* 2017;35(2):210–216.
10. Nair D, et al. Prevalence of carbapenem-resistant Gram-negative bacilli in Mumbai. *Indian J Med Res.* 2021;153(5–6):673–680.
11. Indian Antimicrobial Resistance Surveillance Network (AMRSN) Annual Report. *Indian Council of Medical Research.* 2021.
12. Singh S, et al. Bacteremia caused by carbapenem-resistant *Acinetobacter baumannii* in Indian ICUs. *Infect Dis Today.* 2023;5(1):15–22.
13. Panchal S, et al. Multicenter study of metallo- β -lactamase producing Gram-negative bacteria in India. *J Clin Diagn Res.* 2017;11(7):DC01–DC05.
14. Kumar R, et al. Tigecycline and multidrug resistance in Gram-negative bacilli from pus and respiratory samples. *J Lab Physicians.* 2024;16(2):101–107.
15. Sharma P, et al. Carbapenem-resistant *Acinetobacter baumannii* and *Klebsiella pneumoniae*: clinical isolates in Indian hospitals. *Int J Infect Dis.* 2022;122:172–180.
16. Gupta V, et al. Demographic and clinical characteristics of carbapenem-resistant Gram-negative infections. *J Clin Diagn Res.* 2022;16(5):DC11–DC17.
17. Franklin C, Liolios L, Peleg AY. Phenotypic detection of carbapenem-susceptible metallo- β -lactamase-producing Gram-negative bacilli in the clinical laboratory. *J Clin Microbiol.* 2006;44(9):3139–3144.
18. 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(9):597–602.
19. Walsh TR, Weeks J, Livermore DM, Toleman MA. Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health. *Lancet Infect Dis.* 2011;11(5):355–362.
20. Queenan AM, Bush K. Carbapenemases: the versatile β -lactamases. *Clin Microbiol Rev.* 2007;20(3):440–458.