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Phenotypic Detection of Metallo- β -Lactamase Production and Antimicrobial Susceptibility Patterns Among Multidrug-Resistant *Klebsiella* spp. Isolated From Intensive Care Units: A Prospective Observational Study

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Abstract

Background

The rapid emergence of multidrug-resistant (MDR) and metallo- β -lactamase (MBL)-producing *Klebsiella* species presents a critical therapeutic challenge in critical care settings. These opportunistic pathogens significantly compromise treatment outcomes in intensive care units (ICUs) by disabling last-line carbapenem antibiotics. This study was conducted to evaluate the age, gender, and ICU-wise distribution of *Klebsiella* spp. clinical isolates, determine their antimicrobial resistance profiles, and compare the efficacy of three phenotypic screening methods for MBL detection.

Material and methods

This prospective, observational study was conducted from December 2023 to December 2025 in the department of microbiology, in collaboration with the ICUs of a tertiary care hospital. A total of 146 non-duplicate *Klebsiella* spp. isolates recovered from various clinical specimens (including endotracheal tube aspirates, urine, sputum, blood, and pus) were included. Identification was performed using standard biochemical profiles, and antimicrobial susceptibility testing (AST) was carried out via the Kirby-Bauer disc diffusion method following Clinical and Laboratory Standards Institute (CLSI) guidelines. All 146 MDR *Klebsiella* spp. isolates were evaluated using three phenotypic methods for MBL detection: the imipenem-EDTA (ethylenediaminetetraacetic acid) combined disc test (CDT), the ceftazidime-EDTA CDT, and the imipenem-EDTA double-disc synergy test (DDST).

Results

Among the 146 isolates, 92 (63.01%) were obtained from male patients, and the highest number of isolates was observed in the 41-60-year age group (55/146; 37.67%). The medical intensive care unit (MICU) was the ICU with the highest number of isolates, accounting for 96 (65.75%) of the 146 isolates. The antimicrobial susceptibility results showed high resistance to several tested antibiotics. The imipenem-EDTA CDT yielded 111 positive results, the ceftazidime-EDTA CDT yielded 102, and the imipenem-EDTA DDST yielded 95 positive results. These findings represent the observed results of the respective phenotypic methods and do not establish their diagnostic sensitivity or superiority.

Conclusion

The study describes the antimicrobial susceptibility patterns and phenotypic MBL detection results among MDR *Klebsiella* spp. isolated from ICU patients. The imipenem-EDTA CDT produced the highest number of positive results among the three evaluated methods. However, the findings do not establish diagnostic sensitivity or superiority because no independent reference standard or molecular confirmation was used. Further evaluation using an appropriate reference standard is needed.

Categories: Infectious Disease

Keywords: antimicrobial susceptibility, carbapenem resistance, intensive care units, klebsiella spp, metallo- β -lactamases, multidrug resistance, phenotypic detection

Introduction

Klebsiella species are Gram-negative, non-motile, encapsulated, rod-shaped bacteria belonging to the *Enterobacteriaceae* family and are widely recognized as significant pathogens in healthcare environments. In critically ill and immunocompromised individuals, they frequently cause severe nosocomial complications, including pneumonia, septicemia, urinary tract infections, and wound infections. The clinical management

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of these infections is increasingly complicated by the emergence of multidrug-resistant (MDR) *Klebsiella* strains that produce metallo- β -lactamases (MBLs), enzymes that contribute to resistance against beta-lactam antibiotics, including carbapenems [1]. MBLs are highly potent, zinc-dependent enzymes that hydrolyze a broad spectrum of beta-lactam antibiotics, including carbapenems, which have historically served as last-line therapeutic agents for severe Gram-negative bacterial infections [2].

The proliferation of MBL-producing *Klebsiella* species is a concern in healthcare settings because it can limit available antimicrobial treatment options [3]. Intensive care units (ICUs) serve as primary epicenters for the transmission and acquisition of these resistant pathogens, exacerbated by the high vulnerability of critically ill patients, prolonged lengths of stay, and the heavy selective pressure exerted by the continuous use of broad-spectrum antimicrobials [4].

To effectively combat these aggressive infections, rapid and precise laboratory identification of MBL production is an absolute necessity. While genotypic molecular assays can definitively pinpoint resistance genes, their high cost and technical demands make them inaccessible for routine, daily use in many clinical microbiology laboratories; consequently, laboratories rely heavily on highly accessible phenotypic detection methods [5]. These phenotypic screening techniques are practical, cost-effective, and fundamentally rely on the functional inhibition of MBL enzymes by metal-chelating agents such as ethylenediaminetetraacetic acid (EDTA), utilizing tests like the combined disc test (CDT) or the double-disc synergy test (DDST) [6-9].

Phenotypic methods are used in clinical microbiology laboratories to screen for MBL production; however, the proportions of positive results may differ between methods. Comparing the observed results of these methods in a local clinical setting may help describe their performance among ICU-associated *Klebsiella* spp. isolates. Therefore, this study aimed to describe the demographic, ICU-wise, and specimen-wise distribution of *Klebsiella* spp. isolates, assess their antimicrobial susceptibility patterns, and compare the results obtained using imipenem-EDTA CDT, ceftazidime-EDTA CDT, and imipenem-EDTA DDST. The study did not establish diagnostic sensitivity or specificity because an independent reference standard was not used.

The primary objective of this study was to assess phenotypic MBL production among MDR *Klebsiella* isolates recovered from ICU patients and to compare the results of three phenotypic MBL detection methods: imipenem-EDTA CDT, ceftazidime-EDTA CDT, and imipenem-EDTA DDST. The secondary objectives were to describe the demographic, ICU-wise, and specimen-wise distribution of the isolates and their antimicrobial susceptibility patterns.

Materials And Methods

Study design and ethical clearance

This prospective, observational study was conducted from December 2023 to December 2025 in the department of microbiology, in collaboration with the ICUs of a tertiary care hospital. Prior to initiation, the institutional review board and ethics committee evaluated and approved the study protocol (Approval number: KVV/IEC/05/2024). As the research focused exclusively on clinical bacterial isolates obtained during routine diagnostic workups and did not involve direct patient contact, interventions, or the disclosure of personal identifiers, the requirement for individual written informed consent was waived by the ethics committee.

Isolation and identification of MDR *Klebsiella* species

The study included non-duplicate clinical isolates of MDR *Klebsiella* species recovered from patients admitted to various ICUs, including the medical, surgical, and neonatal ICUs. Clinical specimens processed included endotracheal aspirates, central venous catheter tips, blood, urine, and deep wound exudates. Non-duplicate MDR isolates recovered during the study period (December 2023 to December 2025) were included, while duplicate isolates were excluded to avoid repeated inclusion of the same isolate.

Initial isolation was performed on MacConkey agar and blood agar plates obtained from HiMedia Laboratories, Mumbai, India. Species identification was performed using standard biochemical tests, including carbohydrate fermentation, urease production, and citrate utilization. No automated identification system was used. Quality control was performed for biochemical identification tests only.

Multidrug resistance was defined as non-susceptibility to at least one agent in three or more antimicrobial categories. Antimicrobial susceptibility testing was performed using the Kirby-Bauer disc diffusion method on Mueller-Hinton agar obtained from HiMedia Laboratories, Mumbai, India, using antibiotic discs from the same manufacturer. Zone diameters were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) M100 guidelines [7]. Isolates demonstrating resistance or decreased susceptibility to carbapenems, specifically imipenem or meropenem, were selected for prospective MBL screening.

Phenotypic detection of metallo-beta-lactamases

Three convenient, cost-effective phenotypic assays were executed concurrently for all carbapenem-resistant

Klebsiella isolates to evaluate their diagnostic performance. The primary mechanism evaluated across these methods was the restoration of beta-lactam activity via the inhibition of MBLs by EDTA, which chelates the zinc ions required for enzyme function.

The first method evaluated was the imipenem-EDTA CDT. A bacterial suspension adjusted to a 0.5 McFarland turbidity standard was inoculated onto a Mueller-Hinton agar plate. Two 10 µg imipenem discs were placed on the agar surface, and an aliquot of 4 µg of a 0.5 M EDTA solution was added to one of the discs. Following overnight incubation at 37°C, an increase in the zone of inhibition diameter of 7 mm or greater for the imipenem-EDTA disc compared to the imipenem disc alone was considered a positive indication of MBL production [8].

The second method evaluated was the ceftazidime-EDTA CDT, which applied the same chelation principle using a substrate from the cephalosporin class. For this assay, two 30 µg ceftazidime discs were placed on a prepared Mueller-Hinton agar plate inoculated with the test strain. One of the ceftazidime discs was supplemented with 4 µg of 0.5 M EDTA solution. After overnight incubation at 37°C, the zones of inhibition around both discs were measured and compared. A zone diameter expansion of 7 mm or more around the ceftazidime disc containing the EDTA compared to the ceftazidime disc alone confirmed a positive result for MBL activity [8].

The third approach evaluated was the DDST, which evaluated spatial synergy between the beta-lactams and the chelating agent. On a prepared Mueller-Hinton agar plate, a 10 µg imipenem disc and a 30 µg ceftazidime disc were placed at a center-to-center distance of 20 mm away from a blank filter paper disc impregnated with 10 µg of 0.5 M EDTA. After incubation at 37°C for 18 to 24 hours, the plates were examined for any visible enhancement, phantom zone, or bridging enlargement of the inhibition zones of either imipenem or ceftazidime toward the central EDTA-impregnated disc, which was recorded as a positive synergistic match for MBL expression [5,6,8].

A total of 146 MDR *Klebsiella* isolates were evaluated for MBL production using the three phenotypic methods described above. The results of each method were recorded separately. Based on the overall interpretation of the phenotypic test results, 121 isolates were classified as MBL-positive and 25 as MBL-negative.

Statistical analysis

Data were entered and analyzed using Microsoft Excel (Microsoft Corporation, Redmond, WA). Descriptive statistics were used to summarize the study findings as frequencies and percentages.

Results

The age-wise distribution of the 146 *Klebsiella* spp. isolates showed that the largest number of isolates was observed in the 41-60-year age group (55; 37.67%), followed by the 61-80-year age group (41; 28.08%). Regarding sex distribution, 92 (63.01%) isolates were obtained from male patients and 54 (36.99%) from female patients (Table 1).

Age group (years)	M, n (%)	F, n (%)	%
1 to 20	1 (0.68%)	1 (0.68%)	2 (1.37%)
21 to 40	27 (18.49%)	17 (11.64%)	44 (30.14%)
41 to 60	26 (17.81%)	29 (19.86%)	55 (37.67%)
61 to 80	35 (23.97%)	6 (4.11%)	41 (28.08%)
81 to 100	3 (2.05%)	1 (0.68%)	4 (2.74%)
Total (n)	92 (63.01%)	54 (36.99%)	146 (100%)

TABLE 1: Age and gender-wise distribution of *Klebsiella* species.

M = male; F = female; n = number of individuals; % = percentage.

The ICU-wise distribution showed that the largest number of *Klebsiella* spp. isolates were recovered from the medical intensive care unit (MICU) (96; 65.75%), followed by the surgical intensive care unit (SICU) (22; 15.07%), neuro ICU (14; 9.59%), and emergency intensive care unit (EICU) (12; 8.22%). One isolate each (0.68% each) was recovered from the pediatric intensive care unit (PICU) and intensive maternity unit (IMU).

These findings describe the distribution of isolates across the study units; however, the number of isolates alone does not establish a unit as a reservoir of infection or demonstrate transmission between patients (Table 2).

Sr. No.	ICU	n	%
1	MICU	96	65.75
2	SICU	22	15.07
3	Neuro ICU	14	9.59
4	EICU	12	8.22
5	PICU	1	0.68
6	IMU	1	0.68
7	Total	146	100

TABLE 2: Distribution of *Klebsiella* spp. isolates according to the hospital unit of origin.

MICU = medical intensive care unit; SICU = surgical intensive care unit; EICU = emergency intensive care unit; PICU = pediatric intensive care unit; IMU = intensive maternity unit; n = number of isolates; % = percentage.

A total of 146 *Klebsiella* spp. isolates were recovered from various clinical specimens collected from ICU patients. The highest number of isolates was obtained from endotracheal tube (ETT) samples, accounting for 42 (28.77%) isolates, followed by urine samples with 35 (23.97%) isolates. Sputum specimens contributed 23 (15.75%) isolates, while pus and pus swabs accounted for 21 (14.38%) isolates. Blood samples yielded 18 (12.33%) isolates. A smaller number of isolates were recovered from ascitic fluid (4; 2.74%), self-retaining catheter (SRC) tip (2; 1.37%), and drain tip (1; 0.68%). These findings indicate that respiratory specimens, particularly ETT samples, were the most common source of *Klebsiella* spp. isolates among ICU patients (Table 3).

Sr. No.	Specimens	n	%
1	ETT	42	28.77
2	Urine	35	23.97
3	Sputum	23	15.75
4	Blood	18	12.33
5	Pus & pus swabs	21	14.38
6	Ascitic fluid	4	2.74
7	Drain tip	1	0.68
8	SRC tip	2	1.37
9	Total	146	100

TABLE 3: Specimen-wise distribution of *Klebsiella* species isolates from ICUs.

ETT = endotracheal tube; SRC = self-retaining catheter.

Antimicrobial susceptibility testing was performed on 146 *Klebsiella* spp. isolates recovered from ICU patients (Table 4). Ceftazidime and imipenem were reported as having the highest susceptibility, with 146 (100%) isolates susceptible to each antibiotic. Amikacin showed susceptibility in 21 (14.38%) isolates, followed by gentamicin in 17 (11.64%). Lower susceptibility rates were observed for cefotaxime (13; 8.90%), amoxiclav (12; 8.22%), aztreonam (12; 8.22%), meropenem (6; 4.11%), ciprofloxacin (4; 2.74%), ceftriaxone (2; 1.37%), cefuroxime (2; 1.37%), and nitrofurantoin (2; 1.37%). Resistance was reported in 144 (98.63%) isolates for ceftriaxone and cefuroxime, 142 (97.26%) for ciprofloxacin, 140 (95.89%) for meropenem, and

134 (91.78%) for amoxiclav. Ampicillin was not interpreted as a meaningful susceptibility finding because *Klebsiella* spp. are intrinsically resistant to this antibiotic.

Sr. No.	Antibiotics	S	%	R	%
1	Amikacin	21	14.38	107	73.29
2	Amoxiclav	12	8.22	134	91.78
3	Ampicillin	11	7.53	135	92.47
4	Aztreonam	12	8.22	99	67.81
5	Cefotaxime	13	8.90	133	91.10
6	Ceftazidime	146	100	0	0.00
7	Ceftriaxone	2	1.37	144	98.63
8	Cefuroxime	2	1.37	144	98.63
9	Ciprofloxacin	4	2.74	142	97.26
10	Gentamicin	17	11.64	111	76.03
11	Imipenem	146	100	0	0.00
12	Meropenem	6	4.11	140	95.89
13	Nitrofurantoin	2	1.37	34	23.29

TABLE 4: Antibigram of *Klebsiella* species isolates from ICU patients.

S = sensitive; R = resistant; % = percentage.

A total of 146 *Klebsiella* spp. isolates were screened for MBL production using three different phenotypic methods, namely, imipenem-EDTA CDT, ceftazidime-EDTA CDT, and imipenem-EDTA DDST. Among the isolates tested by the imipenem-EDTA method, 111 (76.03%) were found to be MBL positive, while 10 (6.85%) were MBL negative. Using the ceftazidime-EDTA method, 102 (69.86%) isolates were detected as MBL positive and 19 (13.01%) as MBL negative. The imipenem-EDTA DDST method identified 95 (65.07%) isolates as MBL positive and 26 (17.81%) as MBL negative. Among the three phenotypic methods evaluated, the imipenem-EDTA CDT detected the highest number of MBL-producing isolates, followed by the ceftazidime-EDTA CDT and the imipenem-EDTA DDST (Table 5).

Sr. No.	MBL detection method	Positive, n (%)	Negative, n (%)	Total tested
1	Imipenem-EDTA CDT	111 (76.03%)	35 (23.97%)	146
2	Ceftazidime-EDTA CDT	102 (69.86%)	44 (30.14%)	146
3	Imipenem-EDTA DDST	95 (65.07%)	51 (34.93%)	146

TABLE 5: Detection of MBL production in *Klebsiella* species isolate from ICUs by different methods.

EDTA = ethylenediaminetetraacetic acid; CDT = combined disc test; DDST = double-disc synergy test; MBL = metallo-beta-lactamase; n = number; % = percentage.

Discussion

MDR Gram-negative bacilli, particularly *Klebsiella* species, represent an important therapeutic challenge in ICUs. Critically ill patients may be vulnerable to healthcare-associated infections because of prolonged hospitalization, exposure to broad-spectrum antimicrobial agents, and the use of invasive medical devices [9]. *Klebsiella* species can acquire resistance to multiple antimicrobial agents, including beta-lactams and carbapenems, making their detection and antimicrobial susceptibility testing important components of

clinical microbiology laboratory surveillance [10].

The present study evaluated 146 *Klebsiella* spp. isolates recovered from patients admitted to different ICUs, described their specimen-wise and ICU-wise distribution, assessed their antimicrobial susceptibility patterns, and evaluated phenotypic methods for detecting MBL production. Phenotypic detection may provide useful information for laboratory surveillance and infection prevention, particularly in settings where molecular testing is not routinely available. However, the results of phenotypic tests must be interpreted according to the methods used and the limitations of the study design [11].

The largest proportion of isolates was observed in the 41–60-year age group, which accounted for 55 isolates (37.67%), followed by the 61–80-year age group, with 41 isolates (28.08%). These findings describe the age distribution of the patients from whom the isolates were recovered. However, because the study did not establish age-specific population denominators or conduct an independent risk-factor analysis, these proportions cannot demonstrate that middle-aged or older patients had a higher risk of *Klebsiella* infection. Previous literature has discussed the potential contribution of comorbidities, clinical severity, and healthcare exposure to infection susceptibility, but these factors were not independently evaluated in the present study [12].

Of the 146 isolates, 92 (63.01%) were obtained from male patients and 54 (36.99%) from female patients. This represents a higher proportion of isolates from male patients in the study population. Nevertheless, the observed distribution does not establish male sex as an independent risk factor for *Klebsiella* infection because differences in admission patterns, underlying clinical conditions, and other potential confounding factors were not assessed. Comparisons with previously published surveillance findings should therefore be interpreted cautiously [13].

The MICU accounted for 96 isolates (65.75%), followed by the SICU with 22 isolates (15.07%), the neuro ICU with 14 isolates (9.59%), and the EICU with 12 isolates (8.22%). One isolate each (0.68%) was reported from the PICU and the IMU. These figures describe the distribution of isolates across the participating ICU settings. The higher number of isolates from the MICU does not, by itself, establish that the unit was a reservoir of infection or that transmission occurred within the unit. Patient numbers, admission patterns, duration of hospitalization, antimicrobial exposure, and infection-control practices were not independently assessed to determine the reasons for this distribution. Although previous studies have discussed the possible roles of antimicrobial selection pressure and cross-transmission in healthcare-associated infections, these explanations cannot be confirmed from the present data alone [14,15].

ETT specimens accounted for the largest proportion of isolates, with 42 (28.77%), followed by urine specimens, with 35 (23.97%). Sputum accounted for 23 isolates (15.75%), pus and pus swabs for 21 (14.38%), and blood for 18 (12.33%). The remaining isolates were recovered from ascitic fluid (4; 2.74%), SRC tips (2; 1.37%), and a drain tip (1; 0.68%). These findings indicate that ETT and urine specimens were the most frequently represented specimen categories in this study. However, specimen distribution alone cannot establish whether an invasive device caused an infection or facilitated transmission. The available data also do not establish whether every isolate represented a clinical infection rather than colonization. The findings should therefore be interpreted as a description of the sources from which isolates were recovered, rather than evidence of device-mediated causation [16,17].

Antimicrobial susceptibility testing demonstrated high reported resistance to several agents. Resistance was highest for ceftriaxone and cefuroxime (98.63% each), followed by ciprofloxacin (97.26%), meropenem (95.89%), ampicillin (92.47%), amoxiclav (91.78%), and cefotaxime (91.10%). Resistance was also reported for gentamicin (76.03%), amikacin (73.29%), and aztreonam (67.81%). In contrast, the reported results indicated 100% susceptibility to ceftazidime and imipenem. These results describe the antimicrobial susceptibility pattern recorded in the study; however, the reported complete susceptibility to imipenem alongside high resistance to meropenem should be rechecked against the original laboratory records, susceptibility interpretations, and applicable breakpoints before final submission. The present study did not investigate the molecular or biochemical mechanisms responsible for the observed resistance pattern. Therefore, the findings cannot establish the presence of particular carbapenemase enzymes, coexisting resistance mechanisms, or specific resistance pathways. The high resistance proportions nevertheless highlight the importance of reliable susceptibility testing and local antimicrobial resistance surveillance [18,19].

Phenotypic detection of MBL production was evaluated using the imipenem-EDTA CDT, ceftazidime-EDTA CDT, and imipenem-EDTA DDST. All 146 *Klebsiella* spp. isolates underwent testing by each of the three methods. The imipenem-EDTA CDT yielded 111 positive results (76.03%) and 35 negative results (23.97%). The ceftazidime-EDTA CDT yielded 102 positive results (69.86%) and 44 negative results (30.14%), whereas the imipenem-EDTA DDST yielded 95 positive results (65.07%) and 51 negative results (34.93%). Overall, 121 isolates were classified as phenotypically MBL-positive and 25 as phenotypically MBL-negative. The 25 MBL-negative isolates were negative by all three phenotypic methods. As molecular confirmation and an independent reference standard were not used, these findings do not establish the diagnostic sensitivity, specificity, or superiority of any individual method [20,21].

Taken together, the study provides a descriptive account of the distribution and antimicrobial susceptibility patterns of *Klebsiella* spp. isolates recovered from ICU patients and compares the positive results obtained using three phenotypic MBL detection methods. The findings support the importance of continued laboratory surveillance and appropriate interpretation of antimicrobial susceptibility and phenotypic test results. Their implications for treatment decisions and infection-control interventions should be considered alongside clinical information, current laboratory standards, and the limitations of the study. The results do not independently establish transmission pathways, risk factors, or the diagnostic accuracy of the evaluated MBL methods.

Limitations

This study was conducted at a single institution and included isolates from ICU patients; consequently, the findings may not be directly generalizable to other healthcare settings. Molecular testing to identify specific MBL genes, such as blaNDM, blaVIM, or blaIMP, was not performed. In addition, an independent reference standard was not used to assess the diagnostic accuracy of the phenotypic MBL detection methods; therefore, their sensitivity, specificity, and overall accuracy could not be established. All 146 isolates underwent the three phenotypic MBL detection methods. However, molecular confirmation of MBL-associated genes and an independent reference standard were not available; therefore, the diagnostic accuracy of the methods could not be established. The overall classification of 121 MBL-positive and 25 MBL-negative isolates should be interpreted separately from the method-specific positive and negative results. The study was descriptive and did not establish independent risk factors, device-associated causation, or transmission pathways. Long-term clinical outcomes, survival, and treatment responses were also not evaluated. Despite these limitations, the study provides local data on the distribution and antimicrobial susceptibility patterns of *Klebsiella* spp. isolates and the positive results obtained with the phenotypic MBL detection methods.

Conclusions

This study demonstrated a high proportion of antimicrobial resistance among *Klebsiella* spp. isolated from ICU patients. The isolates were predominantly obtained from male patients and patients in the middle-aged and elderly age groups, with the highest number of isolates recovered from the MICU. ETT specimens and urine were the most frequent specimen sources. The isolates exhibited high resistance to several tested antimicrobial agents, including fluoroquinolones and meropenem. Among the three phenotypic methods evaluated for MBL detection, the imipenem-EDTA CDT yielded the highest proportion of phenotypically positive results. However, because no independent reference standard was used, the comparative diagnostic sensitivity and accuracy of the methods could not be established. These findings highlight the importance of routine antimicrobial susceptibility testing, appropriate phenotypic screening for MBL production, infection prevention and control measures, and antimicrobial stewardship in ICU settings. Further studies using an independent reference standard or molecular confirmation are needed to validate the performance of the phenotypic methods.

Additional Information

Author Contributions

All authors have reviewed the final version to be published and agreed to be accountable for all aspects of the work.

Concept and design: Pratignya G. Biradar

Acquisition, analysis, or interpretation of data: Pratignya G. Biradar, Harsha V. Patil, Satish R. Patil

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Supervision: Harsha V. Patil, Satish R. Patil

Disclosures

Human subjects: Informed consent for treatment and open access publication was obtained or waived by all participants in this study. Institutional Ethics Committee, Krishna Vishwa Vidyapeeth (Deemed to be University), Karad issued approval KVV/IEC/05/2024. **Animal subjects:** All authors have confirmed that this study did not involve animal subjects or tissue. **Conflicts of interest:** In compliance with the ICMJE uniform disclosure form, all authors declare the following: **Payment/services info:** All authors have declared that no financial support was received from any organization for the submitted work. **Financial relationships:** All authors have declared that they have no financial relationships at present or within the previous three years with any organizations that might have an interest in the submitted work. **Other relationships:** All authors

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