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Detecting Carbapenem Resistance in *Enterobacteriaceae* Isolates Using Carbapenem Discs and the Modified Hodge Test at a Tertiary Care Hospital in Maharashtra, India

Naik Shraddha Dinkarrao[1*], Ghogare Harish Subhashrao[2], Kulkarni Smita Sitaram[1], Mulay Manjushree Vijay[1]

[1]Department of Microbiology, Mahatma Gandhi Mission's Medical College and Hospital, Aurangabad, Maharashtra, India; [2]Department of Microbiology, Sri Aurbindo Institute of Medical Sciences and Post Graduate Institute, Medical College and Hospital, Indore, Madhya Pradesh, India

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***Correspondence**
Email: shraddhakulkarni781@gmail.com
Tel: +912406601100
Fax: +912402484445

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ABSTRACT

Introduction: The emergence of carbapenem-resistant *Enterobacteriaceae* (CRE) poses a significant public health concern due to its potential for increased mortality and morbidity. The limited availability of effective antibiotics further exacerbates the dissemination of carbapenem-resistant bacteria. This study aimed to evaluate the prevalence of carbapenem resistance and carbapenemase production in *Enterobacteriaceae* isolates using the Modified Hodge Test. **Methods:** This observational study was conducted at the Department of Microbiology, MGM Medical College & Hospital, Aurangabad, Maharashtra, from November 2015 to November 2017. 171 *Enterobacteriaceae* isolates from various clinical samples were comprehensively tested for carbapenem resistance and carbapenemase production. This involved the use of carbapenem discs (ertapenem, meropenem, and imipenem), E-test strips for ertapenem and meropenem, and the Modified Hodge Test (MHT) for carbapenemase identification. **Results:** Among the 171 tested *Enterobacteriaceae* isolates, a substantial proportion (40%) displayed resistance to carbapenems, as determined by disc diffusion and E-test methods. Among the carbapenem-resistant isolates, 13 were positive for the MHT. **Conclusion:** Our study revealed a notable prevalence of carbapenem resistance in *Enterobacteriaceae* isolates from a tertiary care hospital. The MHT, following Clinical and Laboratory Standards Institute (CLSI) guidelines, demonstrated high sensitivity (> 90%) and specificity (> 90%) for detecting KPC-type carbapenemases in these isolates. Treatment options for CRE infections are limited, with tigecycline and colistin identified as potential options. Our study highlights the importance of promptly diagnosing different carbapenemases using PCR techniques. Consequently, we strongly advocate for implementing robust antimicrobial stewardship programs and infection control practices in healthcare settings to prevent CRE spread effectively.

INTRODUCTION

Members of the *Enterobacteriaceae* family play a pivotal role as pathogenic bacteria affecting human health. They are responsible for a wide range of diseases, including but not limited to urinary tract infections, bloodstream infections, and nosocomial infections. Moreover, these bacteria constitute a substantial proportion of isolates obtained from diverse clinical samples [1]. The production of extended-spectrum β-lactamases (ESBLs) by Gram-negative bacteria has been recognized as a paramount concern in clinical therapeutics [2, 3]. Multiple studies have reported a rising prevalence of infections attributed to ESBL-carrying bacteria [3, 4, and 5]. Consequently, the use of β-lactamase inhibitor/β-lactam combinations, monobactams, and carbapenems has increased. Nonetheless, in recent years, there has been a concurrent rise in resistance to these therapeutic agents [6]. Carbapenems represent a class of β-lactam antimicrobial agents characterized by their extensive spectrum of activity. These agents exert their action by

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inhibiting cell wall synthesis. Carbapenems serve as a crucial last-line therapy against numerous multidrug-resistant strains of Gram-negative bacteria, notably *Enterobacteriaceae*, that produce ESBLs and AmpC enzymes [7].

The emergence of CRE poses a substantial threat, given the elevated risks of mortality and morbidity associated with these strains [8]. The dissemination of carbapenem-resistant bacteria has emerged as a critical concern, primarily attributable to the limited availability of efficacious antibiotics to treat infections caused by these pathogens [9]. CRE encompasses *Enterobacteriaceae* strains that resist any carbapenem antimicrobial agent or produce carbapenemase through either genotypic or phenotypic mechanisms [8].

Carbapenem resistance in *Enterobacteriaceae* can arise from various mechanisms. Specific CRE strains possess β-lactamases, including AmpC or extended-spectrum β-lactamases (ESBLs), which can diminish the organism's susceptibility to carbapenems in conjunction with porin mutations. Certain CRE strains harbor carbapenemase enzymes (also called carbapenemase-producing CRE or CP-CRE), which can directly hydrolyze and inactivate carbapenems [8].

Carbapenemases are commonly carried on mobile genetic elements that facilitate the horizontal transfer of resistance genes between *Enterobacteriaceae* and other Gram-negative bacteria [8].

The Clinical and Laboratory Standards Institute (CLSI) has endorsed the Modified Hodge Test as a recommended method for detecting carbapenemase production [10]. MHT is a valuable and cost-effective phenotypic method for identifying carbapenemase production in *Enterobacteriaceae* isolates. It holds particular significance in resource-limited settings where molecular techniques for detecting carbapenemase genes may not be readily available [10].

Given the limited availability of effective antibiotics for treating infections caused by carbapenem-resistant bacteria, it is imperative to prioritize the detection of such pathogens as an urgent need of the hour [9].

This study aimed to detect CRE in a tertiary care hospital by employing carbapenem discs and E-test strips, as well as to identify strains of *Enterobacteriaceae* producing carbapenemases through the utilization of MHT. The MHT is a valuable and cost-effective phenotypic method for detecting carbapenemase production in members of the *Enterobacteriaceae* family, particularly in situations where molecular methods for detecting carbapenemase genes are not accessible.

MATERIAL AND METHODS

This prospective observational study was conducted in the Department of Microbiology following the approval of the Institutional Ethical Committee (IEC Number: MGM/ECRHS/2015/01). The study was carried out between November 2015 and November 2017. The study included a total of 171 isolates of *Enterobacteriaceae* obtained from various clinical samples, including urine (71), pus (56), bile (9), wound swabs (8), endotracheal secretions (7), blood (6), sputum (5), stool (4), cerebrospinal fluid (CSF) (1), ascitic fluid (1), hematoma fluid (1), ear swabs (1), and nasal swabs (1). The sample size for this study was determined through a comprehensive literature review and consultation with a statistician, ensuring appropriate statistical power and representation. The study encompassed all isolates belonging to the *Enterobacteriaceae* family. However, repetitive isolates obtained from the same patient were excluded from the analysis to avoid duplication and ensure independent representation.

Processing of samples. All samples were aseptically inoculated onto 5% sheep blood agar and MacConkey agar plates, followed by aerobic incubation at 37 ℃. The plates were carefully examined for bacterial growth after an overnight incubation period. The organisms were identified using standard laboratory identification methods, which involved analyzing their colony morphology, Gram staining characteristics, and motility and performing various relevant manual biochemical tests. These tests included cytochrome oxidase, catalase, indole, methyl red, Voges-Proskauer, citrate utilization, urease, triple sugar iron agar, decarboxylase, o-nitrophenyl-β-D-galactopyranoside, nitrate reduction, and phenylalanine deaminase tests [11]. Antimicrobial susceptibility testing was conducted using ertapenem, meropenem, and imipenem discs (HIMEDIA, India) through the Kirby-Bauer disc diffusion method [12]. The testing was performed on Mueller-Hinton agar, following the guidelines set by the Clinical and Laboratory Standards Institute (CLSI) [10]. After conducting antimicrobial susceptibility testing, strains demonstrating resistance to any of the three tested carbapenems (ertapenem/meropenem/imipenem) underwent additional screening for carbapenem resistance using MIC E-test strips (HIMEDIA EZYMIC™) [10]. Carbapenem resistance among *Enterobacteriaceae* strains was defined as a minimum inhibitory concentration (MIC) equal to or exceeding 2µg/ml for ertapenem and/or 4µg/ml for meropenem [8]. The isolates exhibiting carbapenem resistance were subsequently subjected to the MHT to ascertain the presence of carbapenemase production [10].

E-Test (Epsilomer-test) [10, 11]. The E-test is a quantitative technique that combines dilution and diffusion principles for susceptibility testing. The E-test strip is carefully positioned onto an agar plate inoculated with the bacterial sample, enabling the antibiotics to be promptly released from the plastic carrier surface and diffused into the agar surface. Following incubation, the growth of bacteria becomes visually discernible as it proliferates, accompanied by the formation of a symmetrical inhibition ellipse along the E-test strip. The MIC value is determined by referencing the scale in µg/ml

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at the precise point where the edge of the inhibition ellipse intersects the E-test strip. The E-test procedure was conducted following the instructions provided by the manufacturer (HIMEDIA EZYMIC™).

The E-test strip was thawed from the freezer and allowed to equilibrate at room temperature for 30 min before the test. Well-isolated colonies of the test strain, obtained from an overnight agar plate, were suspended in saline. The turbidity of the suspension was adjusted to match the 0.5 McFarland standard. Subsequently, a sterile cotton swab was immersed in the inoculum suspension, followed by gently removing excess fluid by carefully pressing it against the inner wall of the test tube. The inoculated swab was streaked across the entire Mueller-Hinton agar (MHA) surface in three directions while rotating the plate at a 60-degree angle. Subsequently, the plate was left to air dry for 5-15 minutes until the agar surface was completely dry before adding the E-test gradient strip. The E-test strip was carefully positioned onto the agar plate, ensuring that the MIC scale faced upward and the highest concentration was placed closest to the rim of the plate. The agar plate was incubated at 37 ℃ for 24 h, during which the zone of inhibition became visible. The reading of the MIC value was conducted at the precise point where the ellipse intersected the scale of the E-test strip.

Interpretation of E-test results. Resistance of *Enterobacteriaceae* strains to carbapenems was defined when the MIC for Ertapenem reached or exceeded 2µg/ml and the MIC for Meropenem reached or exceeded 4µg/ml. For quality control purposes in the E-test, *E. coli* ATCC 25922 was employed [10].

Modified Hodge Test (MHT). Carbapenemase production was detected using the Modified Hodge Test, following the recommendations outlined by the CLSI guidelines [10]. A sterile cotton swab was employed to evenly inoculate the surface of a Mueller-Hinton agar plate with a suspension of *E. coli* ATCC 25922, which had been adjusted to a turbidity equivalent to 1/10 of McFarland 0.5. After a brief drying period at room temperature, a disc containing 10µg of meropenem was centrally placed on the agar plate. Subsequently, carbapenem-resistant test strains, obtained from an overnight culture, were streaked densely, commencing from the periphery of the meropenem disc and extending towards the outer edge of the plate. *Klebsiella pneumoniae* ATCCBAA-1705 was the positive control, whereas K. pneumoniae ATCCBAA-1706 was the negative control for carbapenemase production. A distorted or clover-leaf-shaped inhibition zone was interpreted as an indication of carbapenemase-producing isolates.

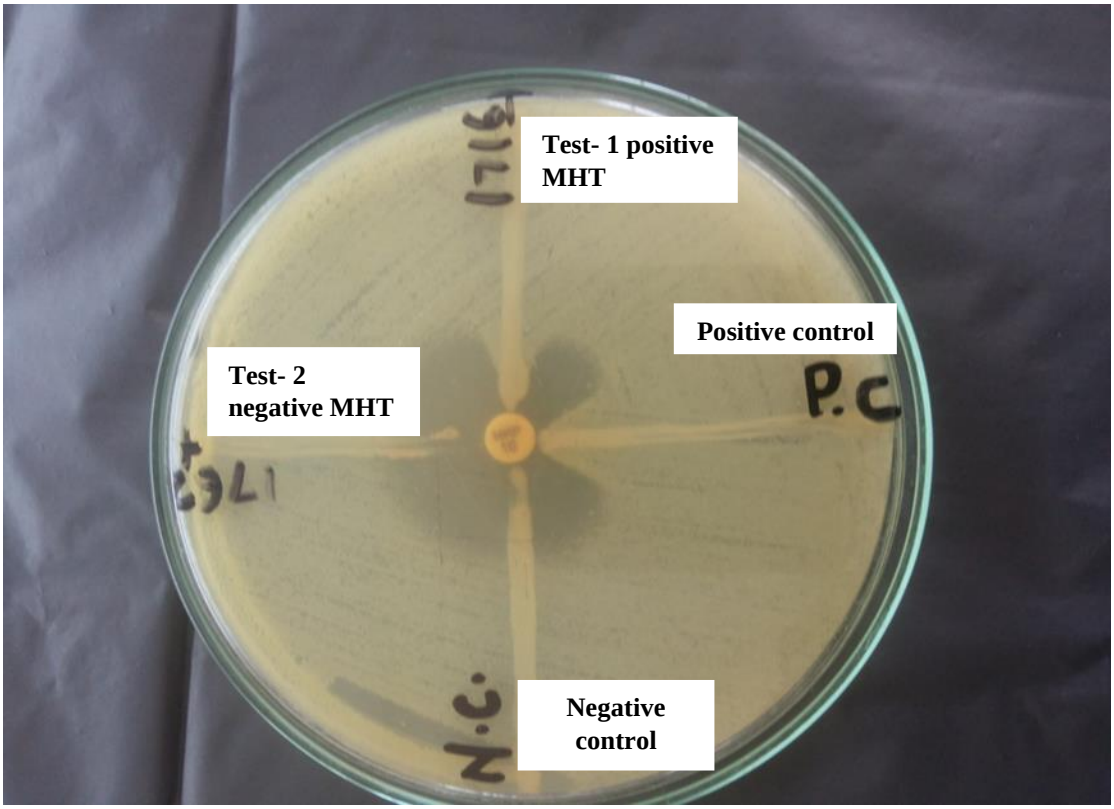


Fig. 1. Modified Hodge Test (MHT). Test 1 presents a positive Modified Hodge Test (MHT) strain exhibiting a distinct cloverleaf-shaped indentation, indicating enhanced *E. coli* ATCC 25922 growth. Test 2 depicts a negative Modified Hodge Test (MHT) result, demonstrating no growth enhancement in *E. coli* ATCC 25922. P.C.: Positive control ATCC BAA 1705 (*K. pneumoniae)*. N.C.: Negative control ATCC BAA 1706 (*K. pneumoniae)*

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RESULTS

Among the 171 *Enterobacteriaceae* isolates included in the study, the majority consisted of *E. coli* (105 isolates, 61.4%), followed by *K. pneumoniae* (57 isolates, 33.33%). Additionally, there were 4 isolates (2.33%) of *Enterobacter cloacae*, 2 isolates (1.16%) of *Enterobacter aerogenes*, and single isolates (0.58%) of *Proteus mirabilis*, *Salmonella typhi*, and *Shigella sonnei*, respectively. Among the 171 *Enterobacteriaceae* isolates analyzed, 22 isolates (12.87%) were sourced from various ICUs, while 149 isolates (87.30%) were obtained from different wards. The sensitivity rates for Ertapenem, Imipenem, and Meropenem were determined to be 61.98%, 60.81%, and 59.64%, respectively. Notably, 69 (40%) of 171 *Enterobacteriaceae* isolates exhibited resistance to carbapenems.

All 69 (40%) of these isolates demonstrated carbapenem resistance as determined by the E-test. Moreover, all isolates that exhibited resistance to meropenem in the disc diffusion test also showed resistance to it in the E-test. Among the isolates, 65 were identified as resistant to ertapenem based on the disc diffusion and E-test techniques. Notably, no inconsistencies were observed between the two screening methods employed.

In the current study, resistance to either Meropenem or Ertapenem was regarded as an indication of CRE. Out of the 69 isolates demonstrating carbapenem resistance, 13 (18.85%) yielded positive results in the Modified Hodge Test (MHT), while the remaining 56 (81.15%) returned negative MHT results. Among the 13 isolates that tested positive for the MHT, 6 (46.15%) were identified as *E. coli*, while 7 (53.85%) were classified as *K. pneumoniae*.

Among the 149 isolates obtained from the ward, 59.06% (n=88) exhibited sensitivity to carbapenems, whereas 40.93% (n=61) were identified as carbapenem-resistant. In contrast, of the 22 isolates obtained from the ICU, 63.63% (n=14) were carbapenem-sensitive, while 36.36% (n=8) were determined to be carbapenem-resistant.

Of the 105 isolates of *E. coli* examined, 68.57% (n=72) displayed sensitivity to carbapenems, whereas 31.42% (n=33) were characterized as carbapenem-resistant. Similarly, among the 57 isolates of *K. pneumoniae*, 40.35% (n=23) exhibited carbapenem sensitivity, while 59.64% (n=34) were determined to be carbapenem-resistant. The percentage of carbapenem resistance was higher among *K. pneumoniae* isolates than *E. coli* in the present study.

Table 1. Antimicrobial sensitivity patterns of all 171 isolates analyzed

Antibiotic	*E. coli* (n=105)		*K. pneumonia* (n=57)		Others (n=9)		Total (n=171)	
	S	R	S	R	S	R	S	R
Ertapenem	74 (70.47%)	31 (29.52%)	24 (42.10%)	33 (57.89%)	8 (88.88%)	1 (11.11%)	106 (61.98%)	65 (38.01%)
Meropenem	72 (68.57%)	33 (31.42%)	23 (40.35%)	34 (59.64%)	7 (77.77%)	2 (22.22%)	102 (59.64%)	69 (40.35%)
Imipenem	74 (70.47%)	31 (29.52%)	23 (40.35%)	34 (59.64%)	7 (77.77%)	2 (22.22%)	104 (60.81%)	67 (39.18%)

Table 2. Distribution of MHT results among a cohort of carbapenem-resistant isolates (n=69)

MHT	No.	Percentage
Positive	13	18.85%
Negative	56	81.15%
Total	69	100%

Table 3. Distribution of carbapenem-resistant and carbapenem-sensitive isolates among patients in both wards and ICUs

Location	Carbapenem		Total	Chi-square	*P*-value
	Sensitive	Resistant			
Ward	88 (59.06%)	61 (40.93%)	149 (100%)	0.031	*P*=0.8606
ICU	14 (63.63%)	8 (36.36%)	22 (100%)		(NS)

NS= Not significant

Table 4. Distribution of carbapenem-resistant and carbapenem-sensitive isolates among *E. coli* and *K. Pneumoniae* bacteria

Isolates	Carbapenem-sensitive No. (%)	Carbapenem-resistant No. (%)	Total No. (%)	Chi-square	*P*-value
E. coli	72 (68.57%)	33 (31.42%)	105 (100%)	12.1	*P*<0.0001(S)
K. pneumoniae	23 (40.35%)	34 (59.64%)	57 (100%)		

S= Significant

DISCUSSION

In our study, the prevalence of carbapenem resistance among *Enterobacteriaceae* was determined to be 40.35%. Notably, in a survey conducted by Mate *et al*. (2014), the prevalence of carbapenem resistance among Gram-negative isolates was reported to be 30%

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(48/160). Among the 48 carbapenem-resistant isolates identified in their study, 31 were classified as *Enterobacteriaceae* [13]. In their investigation, Mulla *et al*. (2011) analyzed the antibiotic sensitivity pattern in *Enterobacteriaceae*, revealing a meropenem resistance rate of 30% among these isolates [14]. Shanthi Mariyappan *et al*. (2017) conducted a study on *Enterobacteriaceae* and reported a prevalence of carbapenem resistance of 45%. Notably, their study employed disc diffusion and minimum inhibitory concentration (MIC) methods, yielding results similar to our findings [15]. Several studies have documented a lower prevalence of carbapenem resistance among *Enterobacteriaceae*. For instance, in a survey by Nair *et al*. (2013), the CRE prevalence was 12.26% [7]. Similarly, Datta *et al*. (2012) documented a prevalence rate of 7.87% for CRE in northern India [16]. Furthermore, Gupta *et al*. (2006) conducted a study where the prevalence of carbapenem resistance in Gram-negative isolates ranged from 17% to 22% [17]. In our study, we identified that 31.42% (33/105) of the *E. coli* isolates were determined to be carbapenem-resistant. Additionally, our findings revealed that 59.64% (34/57) of the *Klebsiella pneumoniae* isolates exhibited resistance to carbapenems. The proportion of carbapenem-resistant isolates was significantly higher in *K. pneumoniae* isolates than in *E. coli* isolates (P=0.0005). This observation aligns with the findings of a study conducted by Kalpana Chauhan *et al*. (2015), which reported carbapenem resistance rates of 14.64% (77/526) among *E. coli* isolates and 29.69% (106/357) among *Klebsiella* spp. isolates [18]. In their study, Wattal *et al*. (2010) documented a higher prevalence of carbapenem resistance in *Klebsiella* species, with rates of 31% in wards and 51% in ICUs, in comparison to *E. coli*, which exhibited rates of 2% in wards and 13% in ICUs [19]. Similarly, Mulla *et al*. (2011) reported a higher resistance rate to meropenem in *Klebsiella* (45.5%) compared to *E. coli* (20%) in their study, which aligns with our findings [14].

In our study, we observed that all isolates exhibiting resistance to Ertapenem/Meropenem as determined by the disc diffusion method also displayed resistance to these antibiotics when tested by the MIC detection using the E-test method. Notably, no discordance was observed between the results obtained from the Kirby Bauer disc diffusion method and the E-test method for detecting carbapenem resistance. Consistent findings have been reported in multiple other studies, including those conducted by Mate *et al*. [13], Datta *et al*. [16], and Shanthi Mariappan *et al*. [15]. In their study, Mate *et al*. (2014) found that all 48 Gram-negative bacterial (GNB) isolates examined exhibited resistance to carbapenems as determined by both the disc diffusion and E-test methods [13]. In their research, Datta *et al*. (2012) observed that all 26 strains displaying resistance to carbapenems according to the disc diffusion method also demonstrated resistance to carbapenems when tested using the E-test method [16]. Similarly, Nagaraj *et al*. (2012) reported no discordance between the two screening methods utilized in their study [20]. Ramalingam *et al*. (2016) reported that among 198 isolates displaying carbapenem resistance by the disc diffusion method in their study, only 117 isolates exhibited resistance to carbapenems as determined by the MIC method using broth dilution [21]. Notably, these findings differ from the observations made in our study.

Our study identified a prevalence of carbapenem resistance in *Enterobacteriaceae* of 40.93% (61/149) among isolates from the wards and 36% (8/22) among isolates from the ICU. Notably, no statistically significant difference was observed between the two groups (P=0.8606).

Our study showed that 18.85% (13/69) of carbapenem-resistant isolates tested positive for the Modified Hodge Test. This proportion is lower compared to other studies conducted by Mate *et al*. (2014), where 60.4% (29/48) of carbapenem-resistant isolates tested positive for the Modified Hodge Test [13], Mahajan *et al*. (2011), who reported that 47.6% of isolates in their study were found to produce carbapenemase based on the MHT [22], and Sathya *et al*. (2015), who reported that 53.50% (61/114) of carbapenem-resistant isolates in their study tested positive for the Modified Hodge Test [23].

In their study, Priya Datta *et al*. (2012) reported that none of the 26 carbapenem-resistant isolates tested positive for the Modified Hodge Test. However, the meropenem-EDTA combined disc test identified 19 isolates as metallo-beta-lactamase (MBL) producers [16].

In a comparative study conducted by Jeremiah *et al*. (2014), it was found that 95.23% (100/105) of carbapenem-resistant *K. pneumoniae* isolates were identified as carbapenemase producers using the Modified Hodge Test (MHT). The study also assessed the performance of the Neo sensitabsCarbapenemase MBL confirmative identification pack, which consists of four types of discs (Meropenem 10 µg [MRP], Meropenem 10 µg + Boronic acid [MR+BO], Meropenem 10 µg + Cloxacillin [MR+CL], and Meropenem 10 µg + Dipicolinic acid [MR+DP]), for the detection of MBL, KPC, and AmpC production [24].

A study conducted by Baran Irmak in Turkey found that 60.8% (110/181) of CRE isolates tested positive for carbapenemase production using the MHT with a Meropenem disc [25].

The CLSI has recommended the MHT for detecting carbapenemases, particularly KPCs, in members of the *Enterobacteriaceae* family [10]. However, it is essential to note that the MHT is not recommended for detecting carbapenemases in non-fermenters [10]. The primary advantage of the MHT is its capability to detect multiple classes of carbapenemases within a

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single plate. However, a significant drawback is its limited ability to differentiate between different classes of carbapenemases, which can result in false-positive results for AmpC and ESBL isolates [26, 27]. In our study comprising 69 CRE isolates, 81.15% (56/69) tested negative for the MHT. The observed carbapenem resistance in these isolates may be attributed to the production of Metallo-β-lactamase (MBL), overproduction of ESBL, or AmpC β-lactamase [8].

Despite the increasing burden of CRE, the optimal treatment for CRE infections remains largely unknown. CRE commonly exhibits resistance to structurally unrelated antimicrobial classes, including aminoglycosides and fluoroquinolones [8, 28]. Limited effective treatment options are available for serious infections caused by CRE. Several studies have reported favorable *in vitro* sensitivity of CRE to tigecycline (ranging from 85.5% to 97%) [18, 23, 29, 30] and colistin (100%) [18, 30]. Hence, these drugs may be considered potential treatment options for CRE infections.

Our study uncovered a noteworthy prevalence of carbapenem resistance among *Enterobacteriaceae* isolates in our hospital, aligning with findings from similar studies conducted in various regions of India. The CLSI guidelines highlight that the MHT exhibits a high level of sensitivity (> 90%) and specificity (> 90%) for the detection of KPC-type carbapenemases in *Enterobacteriaceae* isolates. In our study, the determination of specific types of carbapenemases present in the isolates was limited by the unavailability of PCR testing at our hospital, which represents a constraint of our study. Given the escalating prevalence of carbapenem-resistant *Enterobacteriaceae*, it is crucial to establish a robust antimicrobial stewardship program and adhere to stringent infection control practices [8, 31] to mitigate the transmission of these carbapenem-resistant bacterial pathogens effectively.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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