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## **Carbapenemase Production in Clinical Enterobacteriaceae: A Phenotypic Study using The Modified Hodge Test in a Tertiary Care Setting in West Bengal**

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### **ABSTRACT**

The present study is to investigate the antimicrobial resistance patterns in 100 clinically important Enterobacteriaceae, including 54 *Klebsiella pneumoniae* and 46 *Escherichia coli*, and to estimate the reliability of Modified Hodge Test (MHT) as a method for detecting carbapenemase due to increasing concerns on global emergence of carbapenem resistance among these predominant isolates. Isolates showing high resistance by Vitek-2 were further tested, being the presence of high phenotypic resistance rates for ampicillin-sulbactam, ceftriaxone and ciprofloxacin. Importantly, more than 90% of both isolates were documented as carbapenem-resistant, and for 55% of the total isolates, the MHT results were positive for the presence of carbapenemase. Conversely, significant sensitivity was observed only for a few agents: amikacin (71%) and nitrofurantoin (60%). The findings illustrate the seriousness of the problem of widespread occurrence of carbapenemase-producing Gram-negative rods in the healthcare environment is, and the four agents shown to be appropriate for the empiric treatment of these infections include polymyxin B, nitrofurantoin, fosfomycin and meropenem plus tazobactam. This study recommends that MHT should be routine in screening all isolates yielding intermediate or susceptible carbapenem disc diffusion results in order to limit treatment failure and



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prevent further spread of resistance.

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## INTRODUCTION

**Enterobacteriaceae** are common organisms; in clinical environments, a number of genera, including *Escherichia*, *Klebsiella*, *Salmonella*, and *Shigella*, seem to be significant contributors to opportunistic and primary infections. These species are an important causative agent of Hospital Acquired Infection and pose significant therapeutic challenges. Carbapenem-defying strains now pose serious risks during hospital stays. Often showing up where care is delivered, they link closely to infections caught while receiving treatment. The fact that these drugs form a covalent bond to the protein in bacteria that is essential for them to construct their outermost layer of cells. The penicillin-binding protein (PBPs) bonds parts of the cell wall. Once attached, the drug stops those links from forming properly. Because the structure falls apart, pressure builds inside until the wall bursts. Even if some germs carry enzymes meant to destroy similar medicines, this group often still works. That strength comes from its shape that fits tightly into several types of binding sites on both thick- and thin-walled microbes.

### **Global spread of carbapenemase-producing Enterobacteriaceae:**

**Geographic Hotspots of Prevalence:** The Enterobacteriaceae family's mechanisms of antibiotic resistance (i.e., production of carbapenemase) were found to vary greatly throughout the world, with the highest levels found in India (22%), China (21%), Egypt (19%), Spain (16%), and the USA (15%). This suggests a lack of a coordinated global response, which is another factor contributing to the increasing surge. (Machado et al., 2022; Tadesse et al., 2022)

**Rise of Critical Resistance:** The rise of Carbapenem-Resistant Enterobacteriaceae (CRE) that followed is a serious and growing threat to public health around the world. CRE infections are predominantly caused by the major pathogens, *Klebsiella pneumoniae* and *Escherichia coli* that are important in both burden of infection and resistance, frequently within healthcare facilities. (Kieffer et al., 2016)

**The Increasing Global Threat of Carbapenem-Resistant *Klebsiella pneumoniae*:** One type of *Klebsiella pneumoniae* should not be causing this degree of severe illness in the form of lung infections, bloodstream problems or swelling in the brain; that's very concerning. Stronger ones, such as imipenem or meropenem, are often effective when other antibiotics fail; these drugs typically handle tough gram-



negatives well. Although recently, few strains have resisted these drugs using NDM or OXA-48, making those powerful medicines useless. By 2017, the WHO had placed these unstoppable infections at the highest threat level. In that period, specialists from the CDC in the U.S. pointed out serious concerns about microbes fighting off final-option medicines. Doctors noticed germs fighting medicine more often starting in the late nineteen nineties. In China, one kind of bacteria taken from blood samples resisted a key antibiotic far more by 2018 than it did just over ten years earlier. (Hu et al., 2020)

**Multidrug-resistant *Klebsiella pneumoniae*: Virulence genes influence pathogenicity:** The emergence of multidrug-resistant (MDR) pathogenic bacteria, which is further exacerbated by the presence of virulence factors that are crucial to the pathogenesis of the bacteria, such as *Klebsiella pneumoniae*, is one of these major public health concerns. The virulence of *K. pneumoniae* is fundamentally linked to factors expressed by genes such as *magA*, *rmpA*, *kfu*, *wabG*, *uge*, and *fimH*. (Shen et al., 2023)

In Nepal, several researchers have found that *Klebsiella pneumoniae* that produces carbapenemase is common; however, the majority of previous research's results were primarily based on phenotypic, rather than molecular, characterization of carbapenemase-producing *Klebsiella pneumoniae*. A few writers recently reported that the prevalence of blaOXA-48 genes found in *K. pneumoniae* ranged from almost 10 to 70%. By detecting the blaNDM-2 and blaOXA-48 genes in carbapenemase-producing *Klebsiella* in a tertiary care facility in Nepal, they expected to satisfy this critical need. (Pyakurel et al., 2021)

**Environmental and Clinical Determinants of Carbapenemase Production (CP) in Enterobacteriaceae:** There is an increasing threat of Carbapenemase-Producing (CP) Enterobacteriaceae, as evidenced by a recent finding of 14.4% CP production in Enterobacteriaceae in Punjab, Pakistan. This high level of antibiotic resistance results from antibiotic pressures in the hospitals, which impose strong selective pressure on resistance genes. Additionally, there is increasing concern of the potential non-clinical transfer of carbapenem resistance that can be facilitated from the consumption of fishery-related products, as several reports described plasmid-mediated carbapenem-resistant bacteria carried by these food sources. (Mustafai et al., 2023) Enterobacteriaceae are frequently multidrug resistant (MDR) with cross-resistance to other important classes of antibiotics such as aminoglycosides, trimethoprim-sulfamethoxazole and fluoroquinolones, seriously limiting the available treatment choices.



This has led to higher death rates and higher treatment expenses than for individuals who cannot produce lactamase. (Sheng et al., 2013)

**Problems and Solutions for Identifying Enterobacteriaceae That Produce Carbapenemase:** An analytical challenge for testing facilities is the presence of carbapenemase-producing Enterobacteriaceae (CPE) with KPC enzyme-related resistance. Minimum Inhibitory Concentration (MIC) values for many enteric bacterial pathogens may fall within the current susceptibility range. In order to address this problem, the Clinical Laboratory Standards Institute (CLSI) released guidelines in January 2009 stating that clinical laboratories should test using disc diffusion tests with smaller inhibition zone diameters and determine positive CPE isolates for CPE production at MIC levels higher than the established susceptible threshold. Because of its high sensitivity and specificity, the Cloverleaf test—also known as the Modified Hodge Test [MHT]—was especially advised for determining the presence of carbapenemase in enteric isolate specimens. (Wang et al., 2011) (Carvalhaes et al., 2010) (Wayne, 2010)

**The Modified Hodge Test (MHT): Principle and Clinical Application:** The Modified Hodge Test, often known as the cloverleaf method, is a technique that uses a change in bacterial activity to determine certain isolates that are resistant. The organism's capability for hydrolysing the antibiotic provides the basis for the test. If a carbapenemase is produced by the tested microbe, the enzyme diffuses into the surrounding agar and renders the carbapenem inactive. The disc's inhibitory impact in that local location is neutralised by this antibiotic breakdown. As a result, the susceptible indicator strain, which the antibiotic often inhibits, is able to "rescue" its growth and spread down the carbapenemase-producer's streak line. Where the streak crosses the zone of inhibition, this accelerated, localised expansion creates a characteristic cloverleaf or keyhole shape, signifying a successful outcome for the generation of carbapenemase. The MHT's great accessibility has made it a foundational screening technique for carbapenem-resistant Enterobacteriaceae (CRE) in clinical settings, though it is being overtaken by quicker and more specific molecular or biochemical techniques. (Pasteran et al., 2016) (Wayne, 2010) (Lee et al., 2003) (Miriagou et al., 2010).

## MATERIALS AND METHOD

The technique of disc diffusion was used to evaluate the antimicrobial sensitivity of gram-negative rods to carbapenemase. Out of the 135 Enterobacteriaceae isolated from various clinical samples collected between January and June 2023, 100 *Escherichia coli* and *Klebsiella pneumoniae* participated in the study.

**Key Study Details and Methodology:****Aspect Details**

<b>Study Period</b>	Six months (January to June 2023)
<b>Total Isolates</b>	135 Enterobacteriaceae; 100 <i>E. coli</i> and <i>K. pneumoniae</i> analyzed
<b>Sample Sources</b>	Blood, pus, urine, tracheal aspirate, wound swab, tissue, body fluid, sputum, etc.
<b>Inclusion Criteria</b>	Consecutive, non-repeat, clinically significant, pure Enterobacteriaceae isolates that were resistant to carbapenems.
<b>Exclusion Criteria</b>	All non-Enterobacteriaceae isolates; samples with suspected improper collection, transport, or contamination.

Each person provided information, which was carefully collected once they provided their consent. Whether a person was seen inside the hospital or outside, it made a difference where they were when samples arrived. The length of stay, particularly in critical care, was also recorded. Alongside previous stays under medical supervision, immune condition was also recorded. Previous surgeries were recorded in files. The conclusion of this specific illness spell was recorded here.

**Sample collection and transport:** Each clinical specimen was collected aseptically and immediately delivered to the lab. Colony counts (CFU/mL) were utilised to direct identification and susceptibility screening after urine was collected as a clean-catch midstream sample in a sterile container. For culturing, blood was drawn straight into broth. In order to prevent contamination, sterile syringes, swabs, or biopsy tools were used to collect pus, exudates, wound swabs, tissue biopsies, bodily fluid aspirates, and sputum. After being received, specimens undergo microscope processing, culture, and initial morphological and Gram stain testing. The isolates were then confirmed biochemically. Further definitive identification of the isolates was achieved through a battery of biochemical tests. The core tests included the IMViC series:

Table 1: Bio-chemical Tests Description

TEST	DESCRIPTION
<b>Indole Test</b>	Indicates the presence of tryptophanase; a pink ring formed after peptone water is added to the reagent of Kovac is a positive result.
<b>Methyl-Red (MR) Test</b>	It determines mixed acid fermentation; a medium which turns red when the indicator (pH) is added is positive.
<b>Voges-Proskauer (VP) Test</b>	Detects the production of acetoin; a positive result of adding VP reagents as a red color.
<b>Citrate-Utilization Test</b>	Assesses the ability to use citrate as the sole carbon source, resulting in an alkaline pH; a color change on Simmon's Citrate slant from green to blue indicates a positive result.
<b>Catalase Test</b>	Detects the catalase enzyme; the rapid production of gaseous bubbles upon adding 3% is positive.
<b>Oxidase Test</b>	This detects cytochrome c oxidase; a positive result is formed when a violet, or purple color is formed on the filter paper after 10 seconds of incubation with the reagent.

**Antibiotic susceptibility testing:** Kirby-Bauer disc diffusion on Mueller-Hinton agar and the automated Vitek 2 system (ID/AST cards) was used to check the antibiotic susceptibility of all *E. coli* and *K. pneumoniae* in accordance with CLSI 2022 guidelines. Vitek 2 was used to get minimum inhibitory concentrations, and the disc diffusion zones were measured after 24 h incubation using a digital calliper. The isolates were screened on a universal panel comprising of carbapenems, cephalosporin, combinations beta-lactam/beta-lactamase inhibitors, aminoglycosides, quinolones, colistin, nitrofurantoin and fosfomycin.

**Modified Hodge Test (MHT) for Carbapenemase Detection:** The Modified Hodge Test (MHT) is a phenotypic assay for detecting carbapenemase-producing *E. coli* and *K. pneumoniae* isolates with reduced carbapenem susceptibility. A McFarland-standardized suspension of the susceptible indicator strain *E. coli*

ATCC 25922 is diluted and spread uniformly on a Mueller-Hinton agar plate. A meropenem disc is placed in the centre, and the test isolates, together with positive (ATCC BAA-1705) and negative (ATCC BAA-1706) controls, are streaked in straight lines from the disc edge to the plate margin. After 24h incubation, carbapenemase-positive strains inactivate the drug, permitting indicator growth toward the disc and forming a characteristic cloverleaf-shaped indentation in the zone of inhibition, which is read as a positive result; absence of this pattern indicates a negative result. (Pasteran et al., 2016)

## Result

**Morphological and Microscopic Characteristics of isolates:** The *E. coli* isolate made red-pink colonies with a halo of acid-precipitated bile on MacConkey agar. The pink colour comes from the fermentation of lactose, and the red colour comes from the bile being neutralised. By comparison, the *Klebsiella* isolate also fermented lactose, producing pink colonies, however, with very large colonies with a thick capsule. These two organisms have a similar appearance of Gram-negative rods, but the mucoid morphology of *Klebsiella* makes it differentiated compared to the smoother and non-mucoid *E. coli* colonies before the biochemical confirmation. [Figure 1]

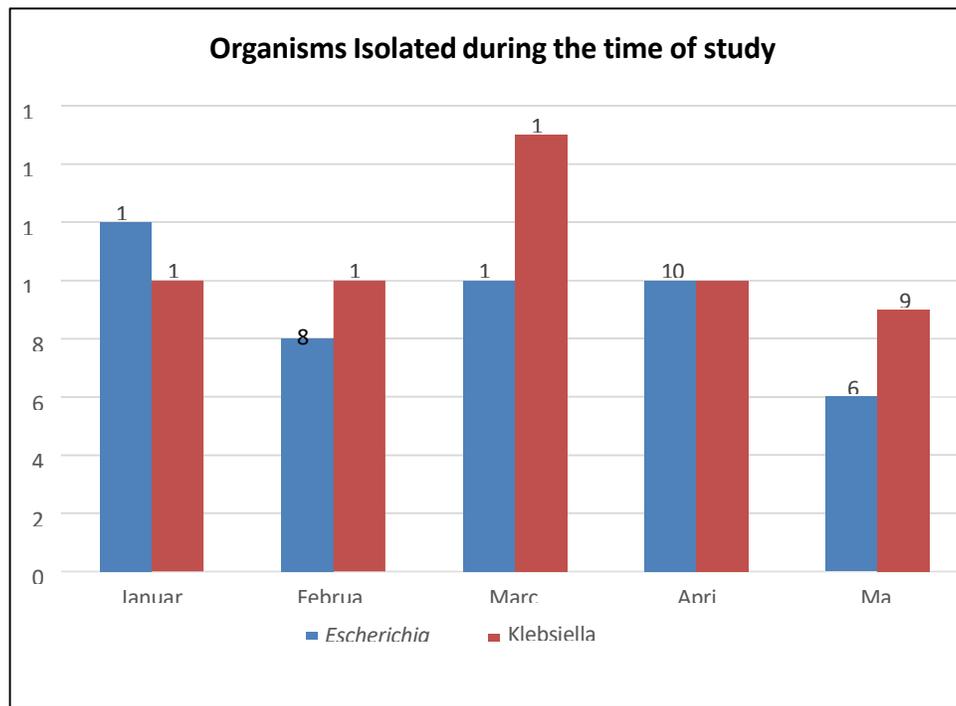


**Figure 1: Isolated Sample cultures on MacConkey Agar Plates**

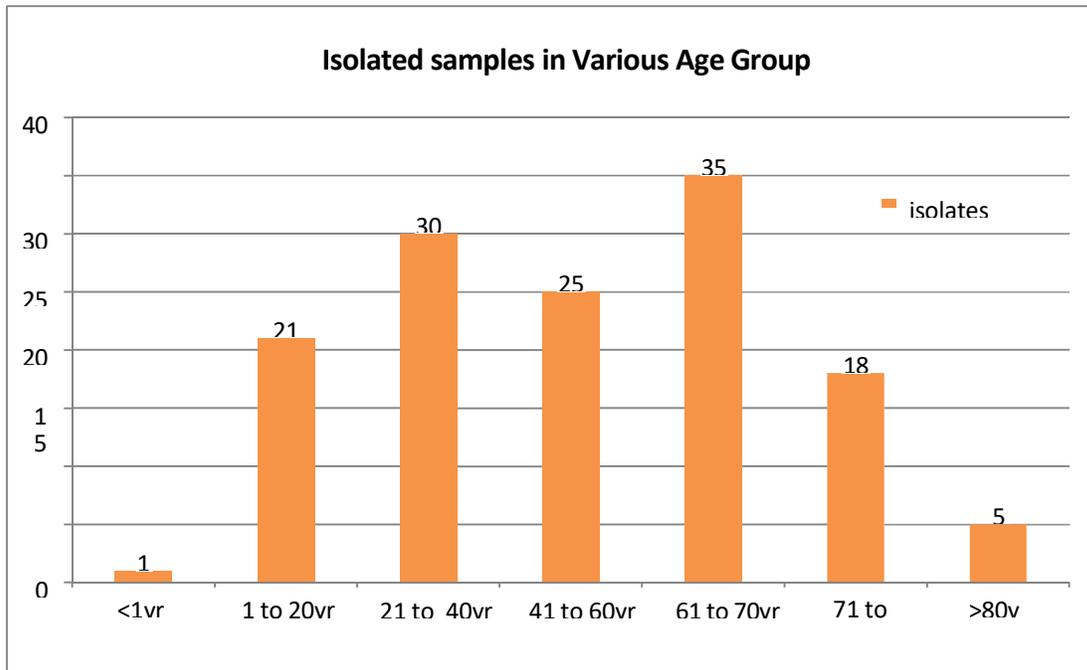


**Pathogen distribution during the study period:** Two species predominated among the 135 isolated Enterobacteriaceae studied over the span of the five-month study period: *Escherichia coli* (46) and *Klebsiella pneumoniae* (54). The majority of the isolates from various clinical samples were these multidrug-resistant organisms, and *Pseudomonas* species were also prevalent. [Figure 2].

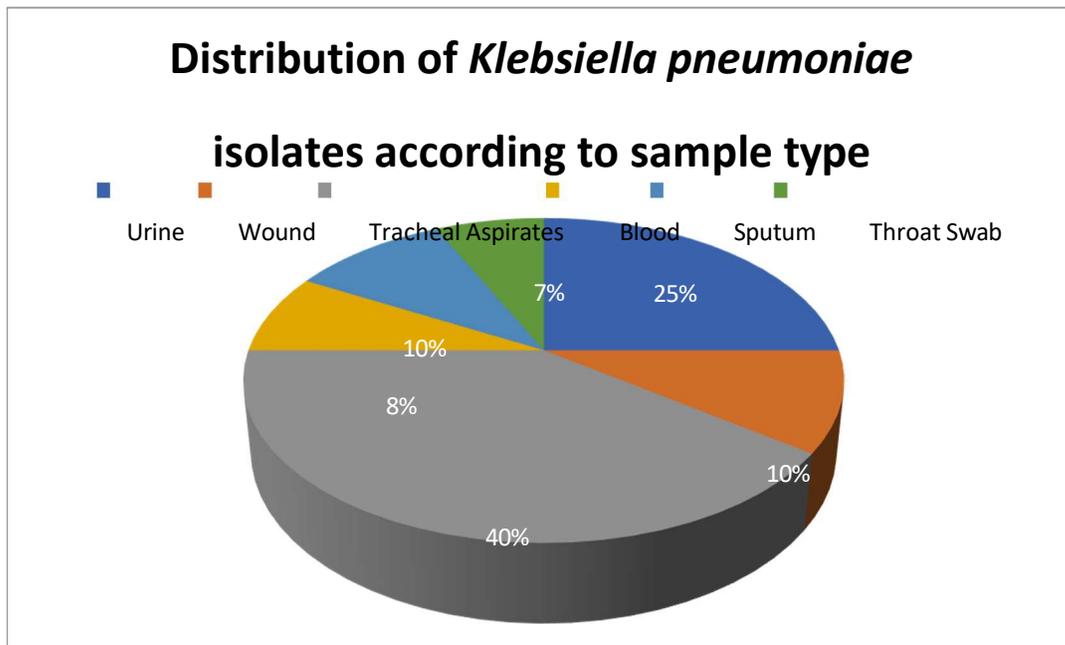
Samples were collected from patients across seven age groups, providing insight into infection distribution by age. Most isolates were from patients aged 61–70 years, followed by those aged 21–40 years. Urinary tract and wound infections showed the highest levels of resistance among all infection types. [Figure 3]



**Figure 2: Total number of pathogens isolated during five months**

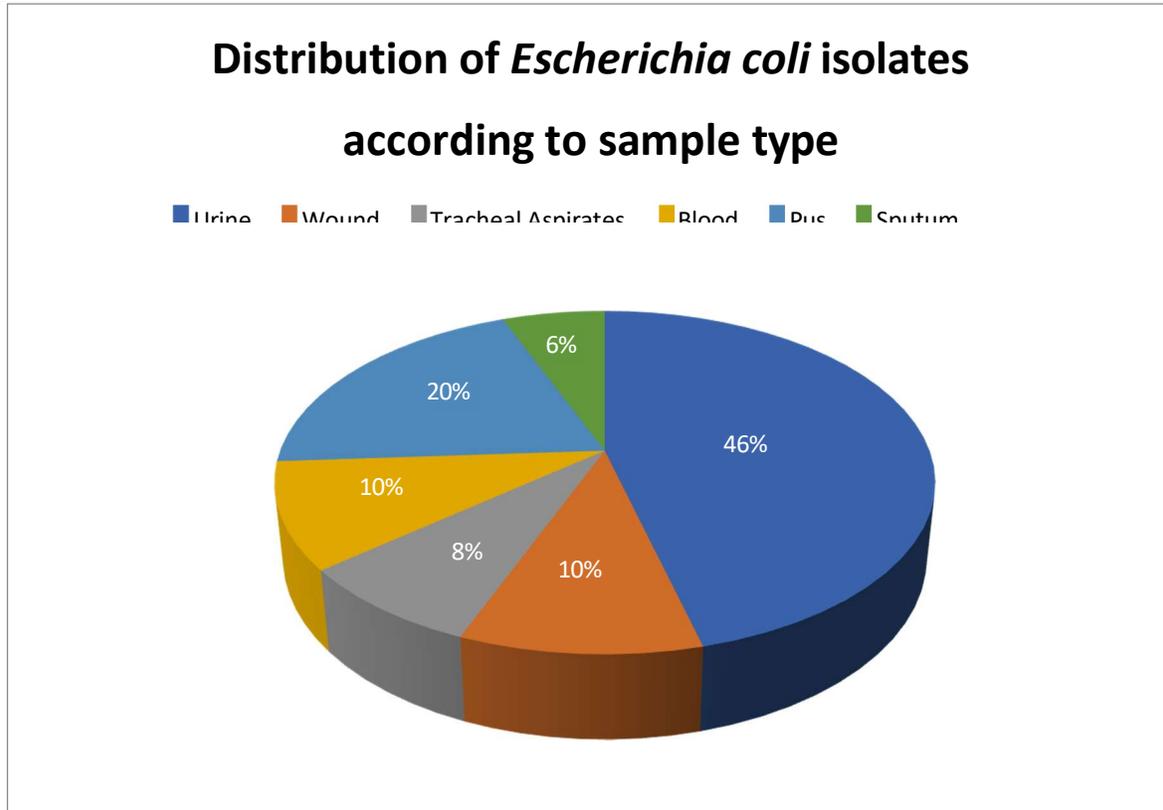


**Figure 3: Age-wise distribution of samples of patients admitted in hospitals.**



**Figure 5: Distribution of K. pneumoniae isolates according to sample source**

**Figure 4: Distribution of *E. coli* isolates according to sample source.**



**Biochemical characteristics of isolates:** The isolates were successfully identified by biochemical test as being specifically Indole-positive (producing a cherry-red ring via tryptophanase) and Methyl-Red (MR)-positive (retaining stable acid products). In contrast, was uniquely Voges-Proskauer (VP)-positive (indicating acetoin production via the butylene glycol pathway) and Citrate utilization-positive (using citrate as its sole carbon source). Both species were confirmed to be Catalase-positive. These distinct profiles established the identity of the two pathogens. The biochemical test result of the isolates is tabulated in [Table 1].

Table 2: Biochemical characteristics of isolates

BIOCHEMICAL TEST	<i>E. coli</i>	<i>K. pneumoniae</i>
Catalase test	+	+
Oxidase test	-	-
Indole test	+	+
Citrate utilization test	-	+
Methyl-red test	+	-

<b>Voges Proskauer test</b>	-	+
<b>Lactose fermentation</b>	+	+

**Antibiotic Resistance and Susceptibility Pattern:** The 100 isolates (*K. pneumoniae* and *E. coli*) performed antibiotic susceptibility testing (AST) using the Vitek 2 automated system and the Kirby-Bauer disc diffusion method (CLSI 2021). A number of drug classes demonstrated extremely high levels of resistance, including ciprofloxacin (90%), ceftriaxone (88%), trimethoprim/sulfamethoxazole (87%), norfloxacin (85%), ampicillin/sulbactam (83%), and piperacillin/tazobactam (81%). With 39 (84.78%) of *E. coli* and 48 (88.8%) of *K. pneumoniae* isolates exhibiting resistance to meropenem, imipenem, and ertapenem, there was concerning evidence of significant resistance to the carbapenem group. In contrast, isolates retained significant susceptibility to select agents: Fosfomycin (83%), levofloxacin (80%), Amikacin (71%), and Nitrofurantoin (65%), suggesting these may be viable therapeutic options. The antibiotic resistance pattern of the isolates is tabulated in [Table 2].



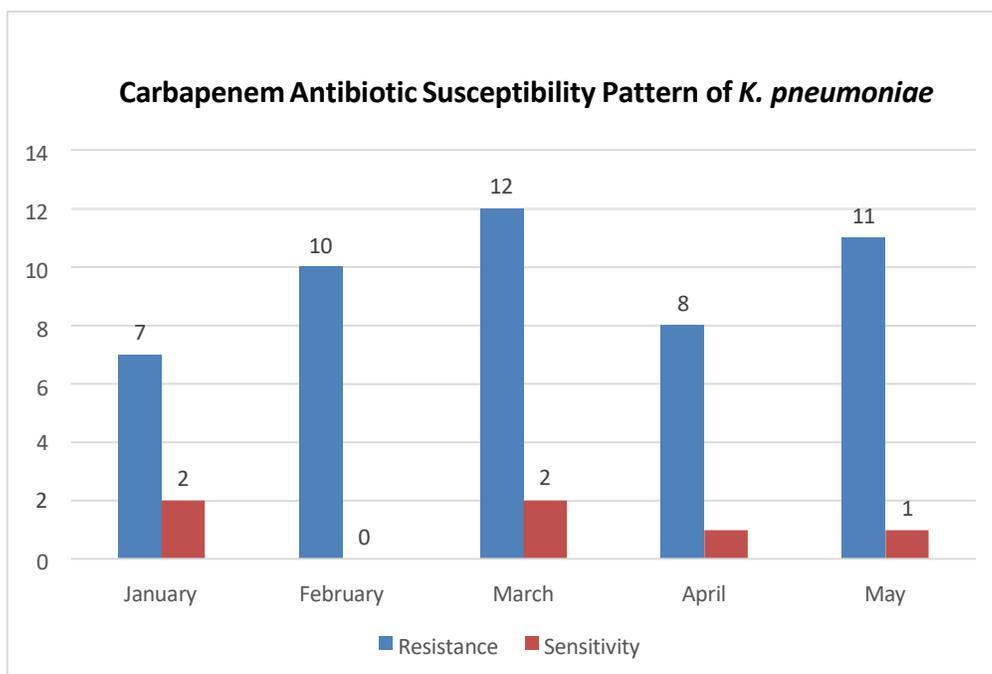
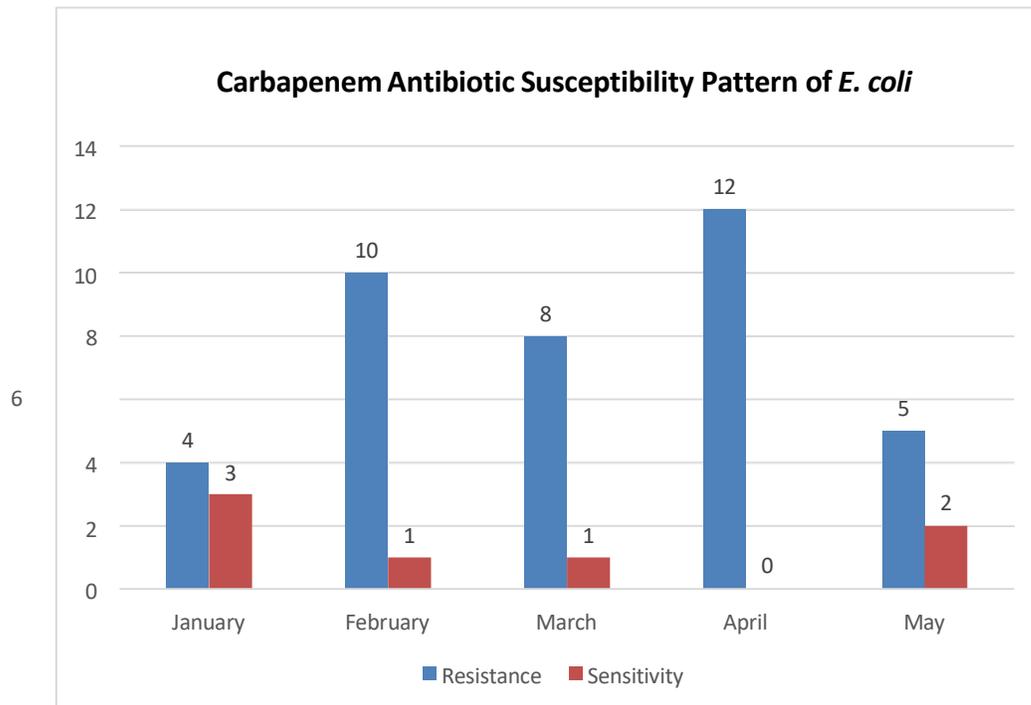
**Figure 6: Antibiotic Susceptibility results by Kirby-Bauer disc diffusion test**

**Table 2: Overall antimicrobial susceptibility pattern of *Escherichia coli* and *Klebsiella pneumoniae* isolated from clinical sources (Jan-June 2023)**

Antimicrobials	Resistance	Intermediate	Sensitivity
Ampicillin/sulbactam	83	1	17



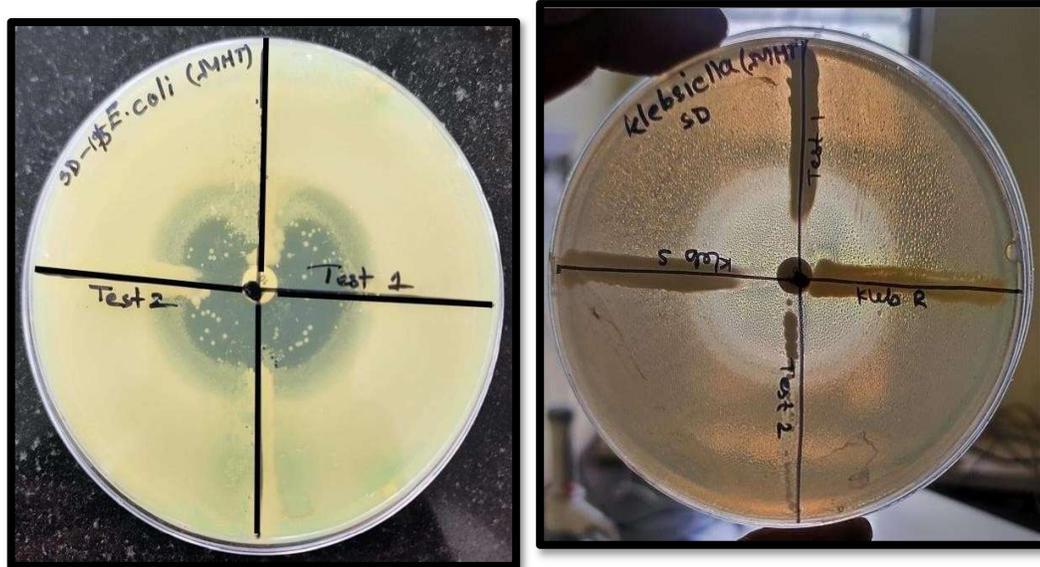
Amoxicillin/Clavulanic acid	89	-	11
Ticarcillin	78	-	22
Piperacillin/Tazobactam	81	-	19
Cefalotin	86	-	14
Cefoxitin	80	-	20
Cefixime	82	-	18
Ceftazidime	85	5	10
Ceftriaxone	88	-	12
Ertapenem	92	3	5
Amikacin	25	4	71
Gentamicin	70	-	30
Nalidixic acid	89	-	11
Ciprofloxacin	90	-	10
Norfloxacin	85	-	15
Ofloxacin	77	-	23
Fosfomycin	17	-	83
Nitrofurantoin	25	10	65
Trimethoprim/ Sulfamethoxazole	87	-	13
Cefepime	16	35	49
Imipenem	91	7	2
Meropenem	95	-	5
Levofloxacin	20	-	80
Colistin	25	43	32



**Figure 7: Susceptibility pattern of *E. coli* and *K. pneumoniae* against Carbapenem.**

**Carbapenemase Production by Modified Hodge Test (MHT):** Out of 100 isolates, 55 were MHT-positive (confirming carbapenemase production) and 32 were MHT-negative. The MHT-positive rate was 76.92% for *E. coli* and 52.08% for *Klebsiella pneumoniae*. A positive result is indicated by a

characteristic cloverleaf-like indentation formed by the growth of the susceptible indicator strain, *E. coli* ATCC 25922, along the test organism's streak within the meropenem disc inhibition zone, signifying antibiotic breakdown. Conversely, a negative result is the absence of indicator strain growth along the streak, demonstrating that the test isolate is not producing carbapenemase and remains susceptible to the carbapenem antibiotic. (Pasteran et al., 2016) [Figure 8].



**Figure 8: Positive result of Modified Hodge Test of isolates with meropenem disc.**

## Discussion

Enterobacteriaceae are not only a part of the normal intestinal flora but also important pathogens in both healthcare-associated (nosocomial) and community settings. Carbapenem-resistant strains can spread easily between humans through various modes such as direct contact, contaminated hands of healthcare workers, and even contaminated food and water. Enterobacteriaceae have a remarkable ability to acquire genetic material, including genes that confer resistance to carbapenemase. This horizontal gene transfer is mainly mediated by mobile genetic elements such as plasmids and transposons. (Partridge, 2011) (Stokes & Gillings, 2011) (Nordmann et al., 2012). Extended-spectrum  $\beta$ -lactamases (ESBLs) are enzymes produced by certain bacteria, including Enterobacteriaceae, that confer resistance to a broad range of  $\beta$ -lactam antibiotics. The high prevalence of ESBL-producing Enterobacteriaceae leads to limited therapeutic options for treating multidrug-resistant (MDR) infections. Long-term hospitalization and the frequent use of invasive medical devices, such as urinary catheters, central venous catheters, and



ventilators, are risk factors for healthcare-associated infections caused by MDR bacteria, including carbapenem-resistant Enterobacteriaceae. (Sabença et al., 2024) (Castanheira et al., 2011).

Global research has evaluated how carbapenem resistance shows up in Enterobacteriaceae. Monitoring resistant strains, figuring out how they show resistance, and shaping treatment choices drive these efforts. The first case of KPC-producing bacteria was first reported in 1996, North Carolina, USA. That moment helped clarify how CRE began spreading. Before 2001, such KPC-carrying microbes rarely turned up. Then came sharp increases across medical centers in New York and New Jersey. (Bratu et al., 2007) (Lomaestro et al., 2006) (Bratu et al., 2005).

In this study, meropenem was identified as an ideal candidate for screening carbapenem-producing bacteria. This is in line with another research that has been published in the past. (Asthana et al., 2014) (Govindaswamy et al., 2019a) (Govindaswamy et al., 2019b)

Our study also revealed high resistance rates against other antibiotics such as cephalothin, cefixime, ertapenem, trimethoprim, and ciprofloxacin. The transmission of this resistance determinants identified in CRE isolates via plasmids or insertion sequences may be the cause of co-resistance to other antibiotic classes of drugs. (Legese et al., 2017).

The 2013 recommendations of the Clinical and Laboratory Standards Institute (CLSI) can be followed for carbapenem resistance screening using zone diameters of ertapenem (10 µg) and meropenem (10 µg). According to these recommendations, a zone diameter of less than 19 mm for ertapenem and less than 16 mm for meropenem is indicative of carbapenem production. (Wayne, 2010) (Hemalatha et al., 2005). The significant finding in your study regarding the high percentage (75%) of isolates showing intermediate or susceptible zone sizes on disc diffusion but testing positive for carbapenemase production by the Modified Hodge Test (MHT) highlights the importance of this simple test in detecting carbapenem-producing organisms. False-positive results can occur, especially in the presence of ESBL production, and confirmatory testing using molecular or genotypic methods may still be necessary to accurately identify carbapenemase enzymes and distinguish them from other resistance mechanisms. (Mohammed et al., n.d.).

The recent advancements in the treatment of CRE have introduced several approved agents, such as ceftazidime-avibactam, meropenem-tazobactam, imipenem-relebactam, cefetecol, and novel aminoglycosides and tetracyclines. Additionally, research is ongoing for phage therapy and the development of new antibiotics. (Tompkins & van Duin, 2021) Polymyxins, such as colistin and polymyxin B, are often used as a last-line treatment for carbapenem-resistant Enterobacteriaceae (CRE)



infections; they do have certain limitations and adverse effects, such as nephrotoxicity and neurotoxicity. (Tran et al., 2016). Tigecycline is effective against the majority of *Klebsiella* isolates, almost all ESBLs, and multidrug-resistant (MDR) *E. coli* isolates. (Shankar et al., 2017).

To address these serious problems, researchers are focusing on creating new antibacterial drugs. Avibactam in combination with ceftazidime is a  $\beta$ -lactamase inhibitor (NB-BLI). (Li et al., 2016). In addition, early implementation of active surveillance through rectal screening for CRE carriage on hospital admission, environmental cleaning, and staff education are all necessary to prevent the spread of outbreaks of bacteria that produce carbapenemase.

## Conclusion

The high percentage of carbapenemase-producing Gram-negative rods in healthcare facilities is a significant concern. Patients who are hospitalized commonly acquire carbapenem resistance, which is mostly brought about by MDR pathogens. The results of this study show polymyxin B, nitrofurantoin, Fosfomycin, and colistin are considered suitable for empirical Enterobacteriaceae treatment in the research area. It is suggested that antibiotic susceptibility be routinely monitored in both clinical and community settings for effective infection control and appropriate antibiotic prescribing. Developing new therapeutic guidelines is indeed an urgent need in the face of CRE infections.

In conclusion, the modified Hodge test is a quick and relatively simple procedure to use to identify bacteria that produce carbapenemase. While the Modified Hodge Test (MHT) may not be considered a specific test for the detection of carbapenem-resistant Enterobacteriaceae (CRE), it can be a valuable tool in resource-limited settings where molecular diagnostic facilities are unavailable. For the benefit of the future, all isolates that show an intermediate or sensitive zone diameter on disc diffusion should be subjected to the modified Hodge test for the production of carbapenemase. As a result, treatment failures and the establishment of antibiotic resistance due to inappropriate usage of this class of drugs will be reduced. By conducting this study, our aim is to gain insights into the antimicrobial resistance profiles and the presence of carbapenemase production in clinically significant *E. coli* and *K. pneumoniae* isolates. This data will contribute to the understanding of the local resistance patterns and the prevalence of carbapenem resistance among these pathogens. It will also help guide appropriate treatment strategies and infection control measures.



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