ISSN: 0975-3583, 0976-2833 VOL15, ISSUE12, 2024

Phenotypic Detection and Molecular Characterization of Carbapenemase Producing Enterobacteriaceae in Clinical Isolates

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Abstract

Aim: The aim of the present study was to detect carbapenemase genes among the carbapenem-resistant isolates in Escherichia coli and Klebsiella pneumonia by phenotypic and molecular methods.

Methods: The current investigation was conducted in the Department of Microbiology in collaboration with the Department of Anaesthesia, Government Medical College Patiala, on 200 isolates of Klebsiella pneumoniae and Escherichia coli from different clinical samples, such as urine, pus, wound, sputum, blood, endotracheal aspirate, pleural fluid, and cerebrospinal fluid, received from indoor patients of Rajindra Hospital, Patiala.

Results: In our study, the maximum number of isolates was from patients aged 41-60 years (38%), followed by 61-80 yrs (36%) and 21-40 yrs (14.50%). The age range was found to be 23 years to 86 years, while the median age was observed as 42 years, and the Mean age was 42.23±18.17. The male-to-female ratio was 1:4.5. 165 cases (82.50%) belonged to the urban background, and 35 cases (17.50%) belonged to the rural background. In this study, the majority of gram-negative isolates was from urine (63.50%), followed by blood (12.50%), pus (11.50%), sputum (7.50%), body fluids (1.50%), tracheal aspirates (1.50%), and Endotracheal tip (1%), and followed by wound swabs (1%). The most common offender was Klebsiella pneumoniae (65%), followed by Escherichia coli (35%) among gram-negative isolates.

Conclusion: The present study revealed a worrisome prevalence of carbapenemase-producing Klebsiella pneumoniae and Escherichia coli isolates. It is essential to detect isolates that are responsible for the production of Carbapenemases to guide antibiotic treatment.

Keywords: Carbapenem-Resistant Enterobacteriaceae, Carbapenemase Producing Enterobacteriaceae, Modified Hodge Test, Double Disk Synergy Test.

1. INTRODUCTION

Members of Enterobacteriaceae family are gram negative, rod-shaped facultative anaerobes mainly colonizing the intestinal tracts of humans and animals and are a common cause of community-associated as well as health care-associated infections.[1] In the past, most of the first line and low-cost antimicrobial drugs such as penicillin and first and second generation cephalosporins have been used effectively for the management of gram-negative bacterial infections. However, drug-resistant organisms acquire resistance to these first-line antibiotics, thereby necessitating the need of second-line drugs like the third and fourth generation

ISSN: 0975-3583, 0976-2833 VOL15, ISSUE12, 2024

cephalosporins. Further emergence of β lactamases, extended spectrum β lactamases (ESBL) 2 and AmpC β lactamases[3] producing bacterial strains lead to the development of multidrug-resistant (MDR) Enterobacteriaceae.

Carbapenems such as ertapenem, imipenem, meropenem, and doripenem have proven efficacy in severe infections caused by these MDR Enterobacteriaceae; therefore, they are frequently used as last resort therapeutic options. Carbapenems have the widest spectrum of antibacterial activities[4] and are also active against the chromosomal cephalosporinases and ESBL, therefore are preferred antibiotics in case of invasive or life-threatening infections. However, in recent years, carbapenem-resistant Enterobacteriaceae (CRE) has emerged in the community as a major health threat. Carbapenem resistance was first reported sporadically in the mid-1990s from the United States and since then reports of carbapenem resistance outbreaks are increasing worldwide at an alarming rate.[5]

In addition to carbapenem resistance, CRE often carry genes that confer high levels of resistance to many other antimicrobials, therefore these bacteria are difficult to treat and are associated with high mortality.[6] The mechanisms of development of carbapenem resistance in Enterobacteriaceae are complex because they involve a broad range of organisms and are mediated by different mechanisms. Broadly, CRE can be carbapenemase producing CRE (CP-CRE) or non-carbapenemase producing CRE (non-CP-CRE).[7] The continuous worldwide spread of carbapenem-resistant Enterobacteriaceae (CRE) is an issue of great clinical and public health concern due to the limited therapeutic options available against infections caused by these organisms.[8] The most common mechanism of carbapenem resistance is the production of carbapenem hydrolyzing β-lactamases (carbapenemase-producing CRE [CPE]). However, other mechanisms contribute to carbapenem resistance, such as overexpression of AmpC, porin loss, and efflux pumps. Three main groups of enzymes are responsible for most carbapenem resistance: Ambler class A (KPC), B (Verona Integron-encoded MBL [VIM], New Delhi metallo-β-lactamase-1 [NDM-1], and IMP), and D (OXA-48-like).[9-10] The rapid and accurate detection of CPE is essential for infection control purposes, especially in nosocomial outbreaks, and may help improve patient management and clinical outcome. Although rapid polymerase chain reaction (PCR) tests for the detection of the major carbapenemase gene families, phenotypic detection may be indicated when molecular methods are not readily available.[11]

The aim of present study was to evaluate efficacy of Modified strip Carba NP (CNP) test against Modified Hodge test (MHT) for early detection of carbapenemase producing Enterobacteriaceae (CPE).

2. MATERIALS AND METHODS

The current investigation was conducted in the Department of Microbiology in collaboration with the Department of Anaesthesia, Government Medical College Patiala, on 200 isolates of Klebsiella pneumoniae and Escherichia coli from different clinical samples, such as urine, pus, wound, sputum, blood, endotracheal aspirate, pleural fluid, and cerebrospinal fluid, received from indoor patients of Rajindra Hospital, Patiala. Escherichia coli and Klebsiella pneumoniae were tested for the prevalence of carbapenemases in Imipenem and Meropenem-resistant isolates by both genotypic and phenotypic methods. The above study was done for a period of 1 Year (Jan 2023 to Dec 2023).

A hospital-based Prospective type observational study was done in the Microbiology department of Patiala in collaboration with the Department of Anaesthesia, Rajindra hospital Patiala.

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Inclusion Criteria

1. The study will include all clinical samples from patients admitted to the ICU of Rajindra Hospital Patiala.
2. This study included only members of the family Enterobacterales, i.e., Escherichia coli and Klebsiella pneumoniae isolates.

Exclusion Criteria

1. Samples both from indoor and outdoor and from other Clinical Departments of Rajindra Hospital Patiala.
2. Other Members of the family Enterobacteriales and Gram-negative bacilli.

Sample Collection

All clinical samples were collected under aseptic precautions by standard procedures and processed according to standard guidelines.

Statistical Analysis

Statistical Package for the Social Sciences (SPSS) software, IBM India; version V26 was used for statistical analysis. The data collected was qualitative and expressed as proportions or percentage. To ensure the results obtained are statistically significant within a 95% level of confidence (with p-value 0.05), systematic hypothesis tests like Z-test and Chi-square test have been performed. Results were analyzed to determine if there was any significant difference in efficiency of MHT and Modified strip CNP test for early detection of CPE. Crosstabs were used to demonstrate the relationship between the two test types MHT and Modified strip CNP test. Cohen's kappa coefficient (κ) was used to measure inter-rater reliability (measure of agreement between two test types).

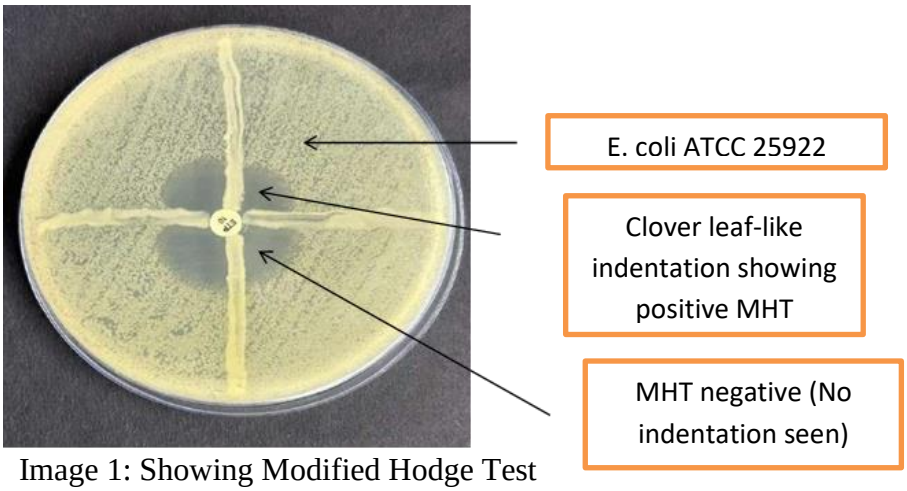

Image 1: Showing Modified Hodge Test

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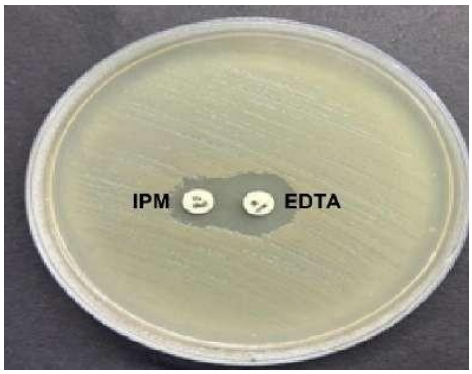

Image 2: Showing IMP-EDTA Double Disk Synergy Test Positive (Synergism Is Seen)

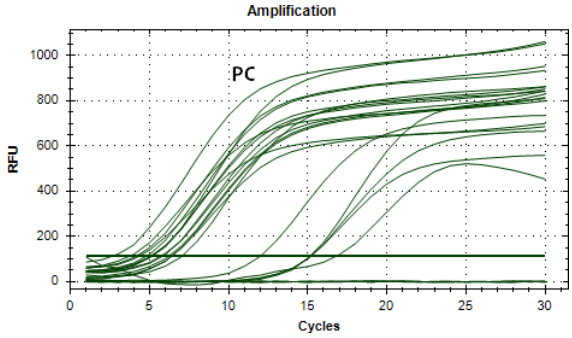

Image 3: Amplification Plot of NDM

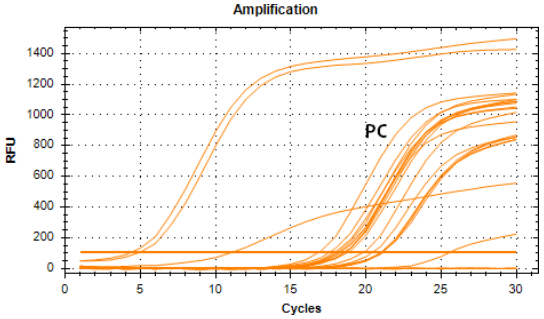

Image 4: Amplification Plot of OXA 23

3. RESULTS

Table 1: Demographic Details

Age Group (Years)	No of Cases	Percentage
0-20	0	0%
21-40	29	14.50%
41-60	78	38%
61-80	72	36%
≥81	2	1%
Gender		
Female	53	26.50%
Male	147	73.50%
Residence		
Rural	35	17.50%

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Urban	165	82.50%

In our study, the maximum number of isolates was from patients aged 41-60 years (38%), followed by 61-80 yrs (36%) and 21-40 yrs (14.50%). The minimum number of isolates was from the > 81 (1%) age group. The age range was found to be 23 years to 86 years, while the median age was observed as 42 years, and the Mean age was 42.23±18.17. 147 (73.5%) were male patients, and 53 (26.50%) were female patients. The male-to-female ratio was 1:4.5. 165 cases (82.50%) belonged to the urban background, and 35 cases (17.50%) belonged to the rural background.

Table 2: Sample Wise and Organism Wise Distribution of 200 Cases

Sample	No of Cases	Percentage
Blood	25	12.50%
Body-Fluids	3	1.50%
ET Tip aspirate	2	1%
Pus	23	11.50%
Sputum	15	7.50%
Tracheal aspirate	3	1.50%
Urine	127	63.50%
Wound Swab	2	1 %
Name of the isolate		
Escherichia coli	70	35%
Klebsiella pneumonia	130	65%

In this study, the majority of gram-negative isolates was from urine (63.50%), followed by blood (12.50%), pus (11.50%), sputum (7.50%), body fluids (1.50%), tracheal aspirates (1.50%), and Endotracheal tip (1%), and followed by wound swabs (1%). The most common offender was Klebsiella pneumoniae (65%), followed by Escherichia coli (35%) among gram-negative isolates.

Table 3: Antimicrobial Resistance Pattern among Escherichia Coli Isolates by Disc Diffusion Method

S.No.	Antibiotic	Escherichia Coli (n=70)	
		S	R
1.	Ampicillin (AMP)	25 (35.8%)	45 (64.2%)
2.	Amoxicillin /clavulanate (AMC)	9 (13%)	61 (87.1%)
3.	Amikacin (AMK)	33 (47.2%)	37 (52.8%)
4.	Gentamicin (GEN)	30 (43%)	40 (57%)
5.	Ciprofloxacin (CIP)	12 (17%)	58 (83%)
6.	Cefuroxime (CXM)	8 (11.5%)	62 (88.5%)
7.	Ceftriaxone (CRO)	20 (28.5%)	50 (71.5%)
8.	Cefotaxime (CTX)	34 (48.5 %)	36 (51.4%)
9.	Ceftazidime (CAZ)	32 (45.7%)	38 (54.3%)
10	Cefepime (FEP)	55 (78.8%)	15 (21.2%)
11	Imipenem (IMI	60 (85.7%)	10 (14.3%)

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12	Meropenem (MEM)	51 (72 %)	19 (28%)
13	Pipercillin/Tazobactam (PTZ)	36 (52%)	34 (48%)
14	Colistin	100 %	-

Out of 70 isolates of Escherichia coli, (64.2%) were resistant to Ampicillin, (87.1%) to Amoxy/clavunate and (48%) showed the Piperacillin-Tazobactam. Escherichia coli shows (52.8 %) and (57%) resistance to Amikacin and Gentamicin respectively. The organism also showed higher level of resistance towards Ciprofloxacin (83%). The organism showed a high degree of resistance pattern against Cephalosporins. Among Cephalosporins, the highest resistance was shown by Cefuroxime (88.5%) followed by Ceftriaxone (71.5%), and Ceftazidime (54.3%) and Cefepime (21.42%). Out of 70 isolates of Escherichia coli, 35 isolates of Escherichia coli showed resistance to one or more extended-spectrum cephalosporins. In the case of the Carbapenem group, the highest resistance was shown towards Meropenem (28%) followed by Imipenem (14.3%). But the organism was 100% sensitive for Colistin.

Table 3: Antimicrobial Resistance Pattern among Klebsiella Pneumoniae Isolates by Disc Diffusion Method

S.No.	Antibiotic	Klebsiella pneumoniae (n=130)	
		S	R
1.	Ampicillin (AMP)	40 (30.7%)	90 (69.3%)
2.	Amoxicillin/clavulanate (AMC)	15 (11.6%)	115(88.4%
3.	Amikacin (AMK)	77 (58.4%)	53 (41.6%)
4.	Gentamicin (GEN)	72 (55.4%)	58 (44.6%)
5.	Ciprofloxacin (CIP)	44 (34.2%)	86 (65.8%)
6.	Cefuroxime (CXM)	18(13.8%)	112 (86.2%)
7.	Ceftriaxone (CRO)	45 (34.6%)	85 (65.4%)
8.	Cefotaxime (CTX)	86 (66.7%)	44 (33.3%)
9.	Ceftazidime (CAZ)	41 (31.5%)	89 (68.5%)
10	Cefepime (FEP)	59 (51.5%)	59 (48.5%)
11	Imipenem (IMI	88 (67.6%)	42 (32.4%)
12	Meropenem (MEM)	85 (65.3%)	45 (34.7) %)
13	Pipercillin/Tazobactam (PTZ)	64 (49.2%)	66 (50.8%)
14	Colistin	100 %	-

In case of Klebsiella pneumoniae out of total of 130 isolates, higher sensitivity was seen towards colistin (100%); In case of Carbapenems group, the highest resistance was shown by Imipenem (32.4%) followed by Meropenem (34.7%), while among cephalosporins, they showed a high degree resistance towards Cefuroxime (86.2%) followed by Cefotaxime (33.3%), Ciprofloxacin (65.8%), Ceftriaxone (65.4%), Cefepime (48.5%), and Ceftazidime (68.5%). Out of 70 isolates, 35 isolates showed resistance to one or more extended-spectrum cephalosporins. Among the Aminoglycosides group, 45 out of 70 isolates of Klebsiella pneumoniae isolates showed resistance to both Amikacin (41.6%) and Gentamicin (44.6%).In case of penicillins group of antibiotics, Amoxyclavunate (88.4%) showed the highest resistance followed by Ampicillin (69.3%), and PTZ (Piperacillin -Tazobactam) showed (50.8%) resistance. Out of 130 Klebsiella pneumonia isolates, Multi-drug resistance was seen in 38

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(29.2%) Klebsiella pneumonia isolates. By the IMP-EDTA Double Disk Synergy Test, MBL production was observed among 50% of Klebsiella pneumoniae isolates.

Table 4: Distribution of Escherichia Coli and Klebsiella Pneumoniae among CRE Isolates and Distribution of CRE Isolates Identified by Modified Hodge Test, Disk Synergy Test, Modified Carbapenem Inactivation Method (Mcim)

Name of the isolate	No of cases	Percentage
Klebsiella pneumoniae	19	73.08 %
Escherichia coli	7	26.92%
Total	26	100%
MHT Test		
Positive	18	69.02 %
Negative	8	30.8%
Total	26	100%
Double Disk Synergy Test		
Positive	17	65.38%
Negative	9	34.67%
Total	26	100%
mCIM Test		
Positive	14	53.8%
Negative	12	46.1%
Total	26	100%

The present study shows that Klebsiella pneumoniae was the most common confirmed CRE isolate (73.07%), followed by Escherichia coli (26.92%). In the present study, Modified Hodge test was performed on all 26 CRE isolates. With Modified Hodge test, 18 cases (69.02%) came out to be positive for Carbapenemase production, and the remaining 8 cases (30.8%) were negative for Carbapenemase. Double Disk synergy test was performed on all 26 CRE isolates. Out of these 17 cases (65.38%) were positive for the DDST test and the remaining 9 cases (34.67 %) were came out to be negative for the DDST test. In the present study, modified Carbapenem inactivation method mCIM method was performed on all the 26 CRE isolates. Out of which 14 cases (53.8 %) were mCIM positive and the remaining 12 cases (46.1%) were negative for mCIM.

Table 5: Distribution of Carbapenemase Positive Isolates by MHT, Double Disk Synergy Test among CRE Isolates

Name of Isolate	No of positive cases	Percentage
Klebsiella pneumoniae	14	77.7%
Escherichia coli	4	22.3%
Total	18	100%
Name of Isolate		
Klebsiella pneumoniae	11	61.22 %
Escherichia coli	6	35.28%
Total	17	100%

18 out of 26 came positive for Carbapenemase production by MHT, where Klebsiella pneumoniae contributed (77.7%) and Escherichia coli (22.3%). 17 came out to be positive for

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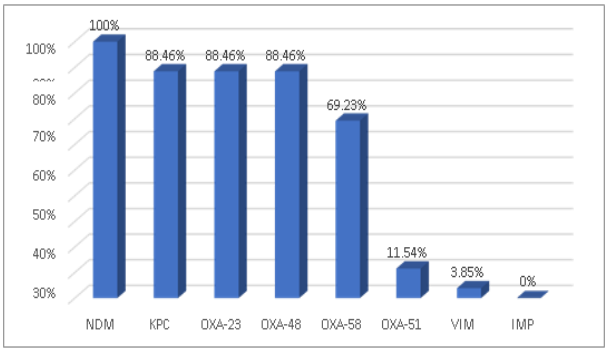
carbapenemase producers by DDST. Klebsiella pneumoniae showed (61.2%) of Carbapenemase production by DDST, whereas Escherichia coli showed 6 (35.28%) Carbapenemase production.

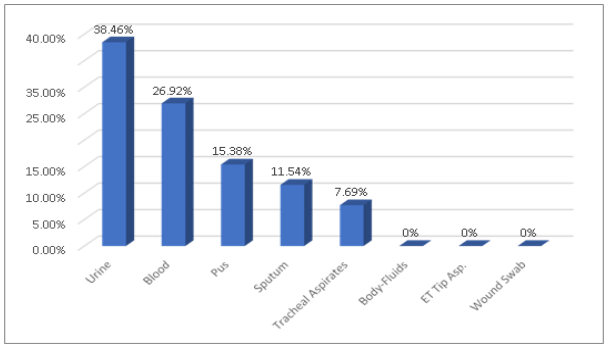
Table 6: Molecular Detection of Carbapenem-Resistant Genes among 26 CRE Isolates

Name of the gene detected	Number	Percentage
NDM	26	100%
KPC	23	88.46%
OXA-23	23	88.46%
OXA-48	23	88.46%
OXA-58	16	69.23%
OXA-51	3	11.54%
VIM	1	3.85 %
IMP	0	0%

In the present study, confirmed CRE isolates (n=26) were further processed for molecular characterization using RT-PCR. In all the CRE isolates (n=26), the NDM gene (100%) was reported, followed by KPC (88.46%), OXA-23 (88.46%), OXA-48 (88.46%), OXA-58 (69.23%), OXA-51 (11.54) %, VIM (3.85) % and IMP (0%).



Graph 1: Molecular Detection of Carbapenem-Resistant Genes among 26 CRE Isolates



Graph 2: Specimen Wise Distribution among Gram-Negative Isolates

(N=26)

In this study, the majority of carbapenem-resistant isolates were from urine samples 10 (38.46%), followed by blood samples 7 (26.9%), pus samples 4 (15.38%), sputum samples 3 (11.54%), and tracheal aspirates 2 (7.69%).

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4. DISCUSSION

Resistance to majority of available antimicrobials has become a major public health problem because the morbidity and mortality associated with these drug-resistant bacterial infections are on the rise all over the world.[12] The highest risk to public health is possessed by the bacteria belonging to the family Enterobacteriaceae, because of their multidrug resistance and rapid dissemination of resistance to other bacterial strains and species.[12, 13]

The emergence of multidrug-resistant organisms at an alarming rate is a cause for concern, as they are responsible for both health care-associated and community acquired infections. The members of the family Enterobacteriaceae are the most commonly implicated organisms of this multidrug resistance. Carbapenems are the drug of choice and in fact the last option left to deal with these multidrug-resistant organisms. However, the ever-increasing resistance to these highly potent agents among Enterobacteriaceae has now spread extensively, mainly as a result of acquisition of carbapenemase-producing genes. Given the frequency with which Enterobacteriaceae cause infections, the high morbidity and mortality associated with these infections and the potential for widespread dissemination of carbapenem resistance through mobile genetic elements, the management of infections caused by CRE is particularly challenging.[14, 15] The present study showed that out of 200 cases, the maximum number of isolates were from patients aged 41-60 years (38%), followed by 61-80 years (36%) and 21-40 yrs (14.50 %). The least number of patients (1%) were found in age group (>80 years old). The study by Nair and Vaz[16] in which most of the CRE isolates were detected in patient samples from the wards (42%) and the ICU (26%) followed by OPD patients (19%).

The Present study showed that out of the 200 cases, the majority of the patients, 147 (73.5%), were male patients, compared to 53 (26.50 %) female patients. Our study shows that out of the 200 cases studied, 165 cases (65%) belonged to the urban background, and 35 cases (35%) belonged to the rural background. In the current study, the majority of gram-negative isolates was from urine (63.50%), followed by blood (12.50%), pus (11.50%), sputum (7.50%), body fluids (1.50%), tracheal aspirates (1.50%), endotracheal tip (1%), and wound swabs (0.50%). In our study, among 200 cases, the most common offender found in gram-negative isolates was Klebsiella pneumoniae (65%), followed by Escherichia coli (35%). In the present study, it was observed that among the 200 isolates, Klebsiella pneumoniae (n=130) was isolated from the majority of the samples as compared to Escherichia coli (n=70). These were subjected to antimicrobial susceptibility testing using the Kirby Bauer disk diffusion method. The studies done by other authors also reported interspecies dissemination of carbapenem resistance like Datta et al[17] reported carbapenem resistance in 62.12% of K. pneumoniae , 7.58% of Klebsiella oxytoca, and 16.6% of E. coli . Bartolini et al[18] reported maximum carbapenem resistance in Klebsiella species (88%) followed by Enterobacter species (8%) and E. coli (4%).

In the present study, Cefuroxime (82%) and Ciprofloxacin (88%) showed maximum resistance towards Escherichia coli. Our study showed that carbapenemase detection by MHT came out around (18/26) (69.2%). MHT positivity in our study consisted of (14/18) (77.7%) for Klebsiella pneumoniae followed by Escherichia coli 4/18 (22.3%). In the present study (IMP-EDTA DDST) was able to detect (17/26) (65.3%) of carbapenemase. Out of which 11/17 (61.22%) came DDST positivity for Klebsiella pneumniae, followed by Escherichia coli (6/17) (35.28%). Thus, Klebsiella pneumoniae was the predominant MBL-producing CRE, followed by Escherichia coli. A plethora of phenotypic tests is available for the detection of carbapenemase-producing organisms with varying sensitivity and specificity. Most commonly used and recommended phenotypic test is MHT.[19-21] It has good sensitivity for the detection of carbapenemase production among organisms harboring KPC and OXA-48 genes. However, it

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requires at least 24–48 h for interpretation of results and it is labor intensive. The other disadvantage of MHT is that false-positive results have been observed among isolates harboring for high-level AmpC or CTX-M gene. Moreover, the results of NDM detection are also not very sensitive. However, the sensitivity for detecting NDM producing organisms can be considerably enhanced on addition of zinc into the culture medium.[22] MHT is recommended as the first-line test for detecting organisms producing carbapenemases. However, MHT cannot discriminate among different types of carbapenemases.[22]

5. CONCLUSION

The present study revealed a worrisome prevalence of carbapenemase-producing Klebsiella pneumoniae and Escherichia coli isolates. It is essential to detect isolates that are responsible for the production of Carbapenemases to guide antibiotic treatment. There is no single phenotypic test that meets all specifications of the "Ideal" test but, there are a number of Molecular options that are user-friendly, accurate, and feasible for implementation in clinical Microbiology Laboratories of all sizes. These findings emphasise the significance of establishing active surveillance networks capable of monitoring and controlling the dissemination of Carbapenemase-producing Escherichia coli and Klebsiella pneumoniae. Reliable detection of Carbapenemase production is essential in combating this problem. Awareness and identification of Carbapenamses are necessary to prevent misreporting and treatment failure. The Antibiotic Stewardship program effectively prevents an increase in MDR Escherichia coli and Klebsiella pneumoniae.

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