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MOLECULAR CHARACTERIZATION OF CARBAPENEM RESISTANT KLEBSIELLA PNEUMONIAE AT A TERTIARY CARE LABORATORY IN KERALA, INDIA

KARAKTERISASI MOLEKULER DARI KLEBSIELLA PNEUMONIA YANG RESISTAN TERHADAP KARBAPENEM DI LABORATORIUM PERAWATAN TERSIER DI KERALA, INDIA

Research Report
Penelitian

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A B S T R A C T

Background: *Isolates of Klebsiella pneumoniae resistant to Carbapenem appears to be most commonly transmitted by hospitalized patients. This may cause infections which lead to mortality. Therefore, studies on molecular level will help to gain more information and be able to know their cross-transmission and resistance mechanisms among these isolates showing resistance.* ***Purpose:*** *Investigate the molecular characterization of carbapenem-resistant Klebsiella pneumoniae isolates from various clinical samples. Klebsiella pneumoniae are aerobic and Gram-negative bacilli found in the gastrointestinal tract of humans and other animals and also as saprophytes in soil.* ***Method:*** *In this study, 401 bacteria of Klebsiella isolates were taken from various clinical samples according to standard protocol. Carbapenem-resistant genes of Klebsiella pneumoniae (CRK) isolates were detected using multiplex polymerase chain reaction (PCR).* ***Result:*** *The twelve isolates resistant to carbapenem were confirmed phenotypically and were confirmed using a multiplex Polymerase Chain Reaction.* ***Conclusion:*** *The present study concluded that identification of Carbapenem-resistant genes of Klebsiella pneumoniae (KPC) could help find out the routes of dissemination of the species and may control its spread within the hospital premises.*

A R T I C L E I N F O

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A B S T R A K

Latar belakang: Isolat *Klebsiella pneumoniae* yang resistan terhadap karbapenem tampaknya paling umum ditularkan oleh pasien yang dirawat di rumah sakit. Hal ini dapat menyebabkan infeksi yang berujung pada kematian. Oleh karena itu, studi pada tingkat molekuler akan membantu mendapatkan informasi lebih lanjut dan mengetahui mekanisme penyebaran lintas dan resistansi pada isolat-isolat yang menunjukkan resistansi. **Tujuan:** Menyelidiki karakterisasi molekuler isolat *Klebsiella pneumoniae* yang resistan terhadap karbapenem dari berbagai sampel klinis. *Klebsiella pneumoniae* adalah bakteri aerob dan Gram-negatif yang ditemukan dalam saluran pencernaan manusia dan hewan lain serta sebagai saprofit di tanah. **Metode:** Pada penelitian ini, 401 bakteri isolat *Klebsiella* diambil dari berbagai sampel klinis sesuai dengan protokol standar. Gen-gen resistan karbapenem dari isolat *Klebsiella pneumoniae* (CRK) terdeteksi menggunakan reaksi berantai polymerase multiplex (PCR). **Hasil:** Dua belas isolat yang resistan terhadap karbapenem dikonfirmasi secara fenotipik dan dikonfirmasi menggunakan reaksi berantai polymerase multiplex. **Kesimpulan:** Penelitian ini menyimpulkan bahwa identifikasi gen-gen yang resistan karbapenem dari *Klebsiella pneumoniae* (KPC) dapat membantu menemukan jalur penyebaran spesies dan dapat mengendalikan penyebarannya di dalam area rumah sakit.

Kata kunci:
Carba NP test, *Carbapenem resistance, Klebsiella pneumoniae, Modified hodge test, Polymerase chain reaction*

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INTRODUCTION

Klebsiella species are found everywhere in nature. They are responsible for both infections that occur in various communities and health care. It comes under gram-negative bacteria that cause a variety of health-related infections and hospital-acquired infections. They can cause sporadic infections or outbreaks. More recently, *Klebsiella* bacteria have shown increasing resistance to a class of antibiotics known as carbapenems. Most clinicians resort to broad-spectrum carbapenems as the last resort for effective treatment (Sekar *et al*., 2016). Antibiotic resistance among *Klebsiella* species has significantly increased and it is associated with significant morbidity and mortality (Tamma *et al*., 2017).

Carbapenem-resistant *Klebsiella* species is spreading all over the world and it has to be looked after seriously (Kumarasamy *et al*., 2010). Identification of carbapenemase-producing at an early stage and the carbapenem-resistant *Klebsiella* is needed to prevent the dissemination of disease within the health care environment (Queenan and Bush, 2007). *Klebsiella* species have a crucial concern both clinically and in public health for the emergence of carbapenem resistance (Nordmann *et al*., 2012).

Mechanisms underlying carbapenem resistance in *Klebsiella* species typically include carbapenemase production and loss or reduced yield of extracellular proteins (Tijet *et al*., 2016). In the present case, the nature of the carbapenem resistance mechanism is not critical to treatment decisions. In the present scenario, the characterization of the mechanism of carbapenem resistance is not crucial for therapeutic decisions. Thus, it is not done as a routine procedure in diagnostic laboratories. The global spread of Carbapenem Resistant *Klebsiella* (CRK) species has turned to be a serious clinical challenge due to limited treatment options for any infection control department, the detection of CRK is critical and its enforcement of intervention at an early stage is a prior concern (Nordmann *et al*., 2012).

The Centers for Disease Control and Prevention has not recommended any production of carbapenemase in a diagnostic laboratory (Mancini *et al*., 2017). Moreover, Weinstein and Lewis (2020) has recommended several findings to trace the production of carbapenemase. Routine microbiology laboratories are limited to resource-rich settings for the detection of molecular detection methods. Hence, this study was done to discover the antibacterial resistance, the prevalence percentage rate of CRK and its phenotypic and genotypic characterization of carbapenemase-producing *Klebsiella* in patients from a Tertiary Care Hospital in Kerala, India.

MATERIAL AND METHOD

Experimental design

Various clinical samples such as pus, urine, sputum, body fluids and blood of the inpatient and out-Patient Department of Microbiology at Sree Gokulam Medical College and Research Foundation, Venjaramoodu, Trivandrum, Kerala, India were processed according to standard protocol and *Klebsiella pneumoniae* isolates were collected. The study was conducted during the period from January 2020 to January 2021. Informed consent was obtained from all the participants in the present study.

Inclusion criteria and exclusion criteria

All the *Klebsiella pneumoniae* isolates from clinical samples such as blood, urine, pus, sputum, wound swab and other body fluids were included. All commensally *Klebsiella* isolates from anatomical sites like gastrointestinal tract, female genital tract, stool and oral throat swab were not included in the study.

Collection of samples

All clinical samples were collected according to standard guidelines and processed according to standard protocol, from various samples, a sum of 401 *Klebsiella* isolates has been obtained. The isolates has been identified with standard microbiological procedures (CLSI, 2015). All *Klebsiella* isolates was checked for their susceptibility to antimicrobial by diagnosing method. An automated system VITEK 2 was used for the identification and diagnosing of antibiotic sensitivity, for the screening of all *Klebsiella* isolates for carbapenem resistance was done by Carba NP test and *Modified hodge* test. Further using conventional PCR method, the carbapenem resistant *Klebsiella* isolates were screened for genes.

Screening test (CLSI recommended disc diffusion test)

A 0.5 McFarland standard inoculum of the test organism was prepared and swabbed onto a prepared Mueller Hinton agar plate, onto which the antibiotic disks of meropenem (10 µg) and ertapenem (10 µg) were placed. After 16 to 18 hours in air, the inhibition zones were measured. Isolates with a zone size of 19 - 21 mm for ertapenem and/or 20-22 mm for meropenem were considered carbapenemase producers.

Modified Hodge Test (MHT)

In this method, a lawn culture of Mueller Hinton agar plate was prepared, into which an overnight culture suspension of *E.coli* ATCC 25922, adjusted to 1:10 dilution of the McFarland 0.5 tube, was added.

In the center, a 10 µg meropenem disc was placed. The test organism was streaked heavily in a straight line from the edge of the disk to its periphery along the culture plate, followed by incubation in a hot air oven at 35 ± 2°C for 16 - 24 hours. The presence of clover leaf-shaped incurvation of *E. coli* strain that was grown around the test streak within the disk diffusion test was considered positive (CLSI, 2016). No zone of *E. coli* strain along the streak of test organism was considered a negative test can be seen in Figure 1.

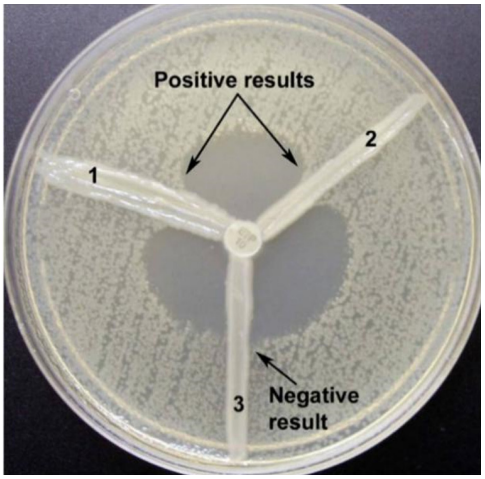

Figure 1. Modified Hodge Test (MHT) positive test showed a clover leaf-like indentation of the *Escherichia coli* 25922 growing along the test organism growth streak within the disk diffusion zone. MHT negative test showed no growth of the *Escherichia coli* 25922 along with the test organism growth streak within the disk diffusion.

Carba NP test

In Carba NP test, (A) solution of phenol red (0.5%) and $ZnSO_4$ (0.1 mmol/L) was prepared with a clinical laboratory reagent water can be seen in Figure 2. The pH of the solution was adjusted to 7.8 ± 0.1. It was stored in amber-colored bottles at 4°C. It was good for the test for up to 15 days. (B) This solution was freshly prepared by adding 1ml of solution A with 6 mg of imipenem-cilastatin powder.

Take two 1.5 ml eppendorf tubes. Label them as 'a' and 'b' and on both, write the bacterial identification number. In tube 'a', add 200 µl of sterile water was added. Emulsify two to three loop full colonies to make a heavy suspension. The suspension should be milky. Aliquot 100 µl from tube 'a' of respective strain to tube 'b'. Add 100 µl of solution A to tube marked 'a' and 100 µl of solution B to tube marked 'b'. Incubate the tubes at 37°C for 2 hours. Reading has to be taken at 30 minutes and 2 hours.

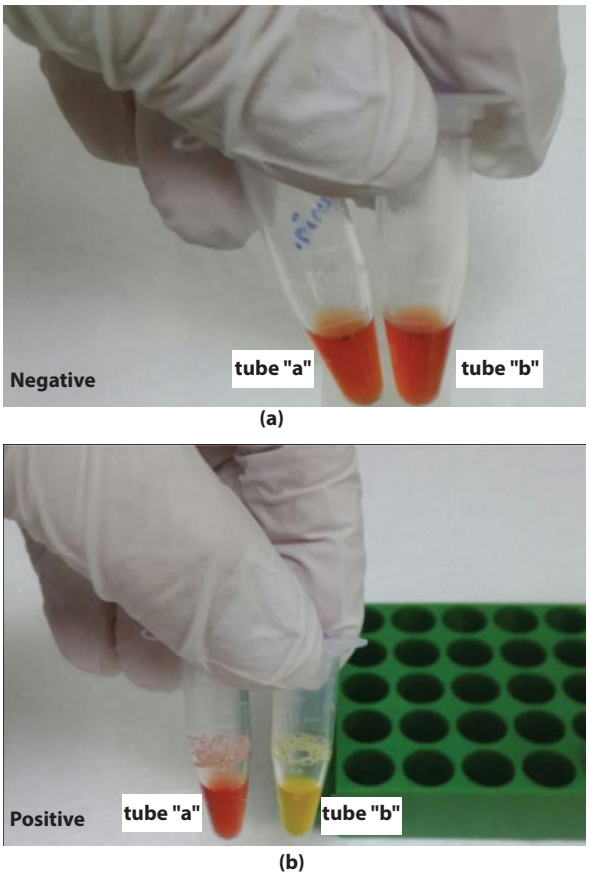

Figure 2. (a) Both tubes remained red in the negative test; (b) Tube "a" was red and tube "b" was orange/yellow; this test was considered positive.

Positive result, the tube "a" becomes red and tube "b" becomes orange/yellow. While if both tubes remained red, it showed the negative test. For positive control, *Klebsiella pneumoniae* ATCC BAA 1705 and negative control, *K. pneumoniae* ATCC BAA 1706 and plain A and B reagents with lysis buffer (reagent control) were used (Rudresh *et al*., 2017; Partridge *et al*., 2018; Pasteran *et al*., 2015).

Gene detection

DNA Extraction: The isolates to be tested were inoculated into sterile nutrient broth. After overnight incubation, the broth was transferred to sterile eppendorf vials and centrifuged at 10000 RPM for 10 minutes. The supernatant was discarded while the pellet was used for the gene detection. The pellet was suspended in 200 µl of PBS and 50 µl of lysozyme was added and incubated at 37°C for 15 minutes. Then 400 µl of Lysis buffer and 40 µl of proteinase K (10 mg/ml) were added and gently mixed well. This was incubated in water bath at 70°C for 10 minutes. The whole lysate was transferred into pure fast spin column and centrifuged at 10000 rpm for 1 minute. The flow through was discarded and 500µl of wash buffer was added and centrifuged at 10000 rpm for 1 minute.

The wash procedure was repeated one more time. The flow through was discarded and the column was centrifuged for additional 2 minutes to remove any residual ethanol. DNA was eluted by adding 100 µl of elution buffer and it was centrifuged for 1 minute. 1µl of extracted DNA was used for PCR amplification.

PCR procedure

The *Polymerase Chain Reaction* (PCR) was set up in a PCR vial, after adding the master mix, the forward and reverse primers and the extracted DNA. 25µl of master mix contained 10X taq buffer, 2mM $MgCL_2$, 0.4 mM dNTPs mix, and 2U proofreading taq DNA polymerase. The primers used were forward primer: 5'-GCT CAG GCG CAA CTG TAA G-3' and reverse primer: 5'-AGC ACA GCG GCA GCA AGA AAG-3'.

The PCR vial was placed in PCR machine and it was subjected to initial denaturation at 94°C for 3 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute and extension at 72°C for 1 minute. A final extension procedure was carried out at 72° C for 5 minutes. Gel electrophoresis was, then, carried out using 2% agarose gel. Gel was viewed in a UV transilluminator and the bands pattern was observed.

Ethical concern

Ethical clearance was obtained from the Ethical committee meeting conducted at Sree Gokulam Medical College and Research Foundation, Venjaramoodu, Trivandrum, Kerala.

Statistical analysis

Microsoft Office Excel 2010 was used for handling and preparing the data. Data were analyzed using SPSS v21 software. The results were expressed as proportions.

RESULT

January 2020 to January 2021, a total of 401 *Klebsiella* isolates from different clinical samples were collected. The distribution of *Klebsiella* isolates in Figure 4 shows the species distribution among various *Klebsiella* isolates. Out of 390 (97%) were identified as *Klebsiella pneumoniae* and 11 (3%) were *Klebsiella oxytoca*.

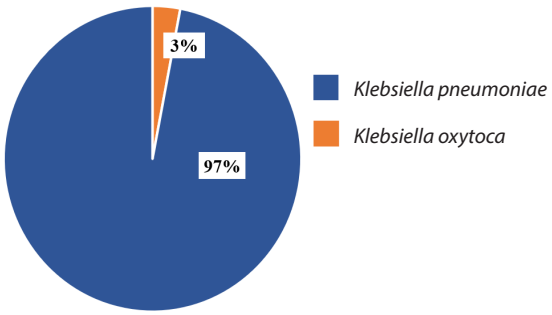

Figure 4. Species distribution

The maximum number of multidrug-resistant *Klebsiella* spp. were isolated from pus sample 21 (44.7%), followed by urine 15 (32%), sputum 8 (17%), and blood 3 (6.3%) can be seen in Table 1. All the multidrug-resistant *Klebsiella* species were subjected to phenotypic characterization for the detection of carbapenemase production. Of the 401 *Klebsiella* isolates, 36 (8.9%) were positive for carbapenemase production in the screening test and confirmation by the MHT and Carba NP test *K. pneumoniae* were the predominant carbapenemase producers can be seen in Table 2. Carbapenemase production among male patients was 9 (75%) and 3 (25%) among female patients. It was noted that a maximum number of cases of carbapenemase producers was noticed in male patients (Table 3).

All the carbapenemase-producing isolates were found to be resistant to three or more classes of antibiotics and are considered as *Multidrug-Resistant* (MDR). They were 100% resistant to ampicillin, cefepime, ciprofloxacin, tetracycline, piperacillin/tazobactam, imipenem, and meropenem. High resistance was also observed against gentamicin (75%), amikacin (70%), and cotrimoazole (83%). Overall antibiogram pattern of the isolates showed a high degree of sensitivity towards colistin (94%).

Table 1. Distribution of multidrug-resistant *Klebsiella* spp. from various samples

Serial No	Samples	Multidrug-resistant *Klebsiella* spp. isolated N=47
1	Pus	21 (44.7%)
2	Urine	15 (32%)
3	Sputum	8 (17%)
4	Blood	3 (6.3%)

Table 2. Phenotypic characterization of *Klebsiella* spp.

Isolates	Number (%)
Total no. of *Klebsiella* spp. Isolates	401
Multidrug resistant *Klebsiella* species	47 (11.72%)
Screening positive *K. pneumoniae*	36 (8.9%)
Modified Hodge test positive *K. pneumoniae*	12 (3%)
Carba NP test positive *K. pneumoniae*	12 (3%)

Table 3. Distribution of carbapenemase producers among male and female patients

Sex	Carbapenemase n = 12	Non carbapenemase n = 389	Total
Male	9 (75%)	202 (52%)	211
Female	3 (25%)	187 (48%)	190

All the CP-CRK were subjected to a mPCR. mPCR for identifying Class A β-lactamases producers (KPC), Class B β-lactamases producers (NDM), and Class D β-lactamases producers (OXA-48) were done. It was noted that 10 isolates expressed KPC followed by one isolate expressing NDM and one isolate expressing OXA-48 (Table 4).

Characterization in molecular level of CP-CRK was done by multiplex polymerase chain reaction (mPCR). All CP-CRK isolates were subjected to molecular characterization. The isolates were grown overnight in a *Tris-EDTA* (TAE) broth. The DNA was extracted by boiling method. The genes were multiplied by using primers. The template DNA, Taq PCR master mix, and primers were added to the PCR mixture (both forward and reverse) shown in Figure 3.

Table 4. Summary of phenotypic and genotypic characterization of CP-CRK

Isolates	Number
Modified hodge test positive *K. pneumoniae*	12
Modified Carba NP test positive *K. pneumoniae*	12
Class *A β-lactamases producers (KPC)*	10
Class *B β-lactamases producers (NDM)*	1
Class *D β-lactamases producers (OXA-48)*	1

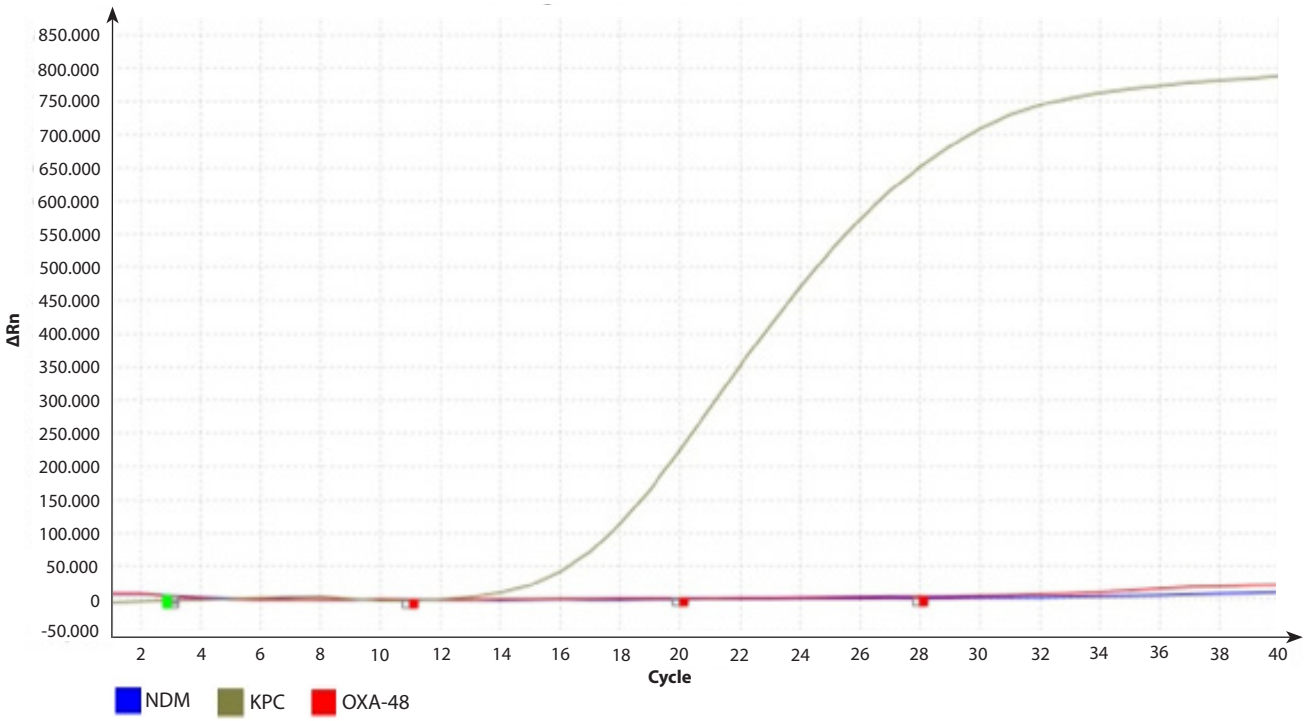

Figure 3. The results of the real-time PCR results for KPC in *Klebsiella*

DISCUSSION

Klebsiella species belong to Enterobacteriaceae, a significant part of the human gut bacteria that can become carbapenem-resistant (Pierce *et al*., 2017). The emergence and dissemination of carbapenem resistance among *Klebsiella* species pose a considerable threat to public health since carbapenems are the agents of last resort in the treatment of life-threatening infections caused by drug-resistant Enterobacteriaceae (Sekar *et al*., 2016).

In the present study, it was discovered that 97% were *Klebsiella pneumoniae* isolates, while 3% were *Klebsiella oxytoca*. This finding was comparable to the reports of Mangayarkarasi *et al*. (2017) and Sridhar *et al*. (2015). *K. pneumoniae* was the predominant species isolated in all the previous reports including the present study. Most of the hospital-acquired urinary tract infections, pneumonia, septicemias, and soft tissue infections are mainly caused by *Klebsiella pneumoniae* other than that genus. Therefore, medically important *Klebsiella* species were taken in this study.

In the present study, the isolation rate of *Klebsiella* spp. was more from pus (44.7%), followed by urine (32%), sputum (17%), and blood (6.3%). Patilaya *et al*. (2019) reported that pus samples were the major source of *Klebsiella* spp. infection followed by sputum samples. According to Ravichitra *et al*. (2014), *K. pneumoniae* isolated from pus, sputum, and urine samples was also resistant to amoxiclav and ofloxacin.

In this study, 11.72% (47/401) of *Klebsiella* spp. was multidrug-resistant. This study was also supported by Ferreira *et al*. (2019) who found that the vast majority (84%, 21/25) of *K. pneumoniae* isolates showed an MDR pattern, either alone or in association with other common antibiotics such as β-lactams (including carbapenems), aminoglycosides, quinolones.

In the present study, carbapenemase producers among *Klebsiella pneumoniae* were 3%. This was also supported by Bina *et al*. (2015) that 14.65% of the *K. pneumoniae* strains were resistant to carbapenems. Of the 401 *Klebsiella* isolates, 12 (3%) tested positive for carbapenemase production in the screening test and were subsequently confirmed by the Modified hodge test and modified carba NP test. *K. pneumoniae* were the predominant carbapenemase producers, no carbapenemase production was detected in *K. oxytoca*. This was also supported by Shanmugam *et al*. (2013) that reported the higher prevalence (82.6%) of Modified Hodge test positive *K. pneumoniae* in India.

All the Carbapenemase-producing isolates were found to be resistant to three or more classes of antibiotics that were classified as *Multidrug-Resistant* (MDR). All carbapenemase positive isolates were 100% resistant to ceftazidime, cefepime, ciprofloxacin, tetracycline, piperacillin/tazobactam, imipenem, and meropenem. High resistance was also observed against gentamicin (75%), amikacin (70%), and cotrimoxazole (83%). Colistin was 94% effective against *K. pneumoniae*. Thus, colistin could be the drug of choice for carbapenem-resistant *K. pneumoniae* isolates. Many researchers (Ansari *et al*., 2021; Shilpakar *et al*., 2021; Shanmugam *et al*., 2013) have also reported high sensitivity against colistin. In this study, more isolates were MDR (11.72%). Many studies have supported that the increase in MDR is due to the easy availability and blind irrational use of antibiotics without proper culture report and prescription (Basnyat *et al*., 2015; Baral *et al*., 2011).

Carba NP test is a biochemical test for rapid detection of carbapenemase production on Gram-negative bacilli. The test has a specificity of 100% and a sensitivity of 84%. This study found that among the 401 isolates, the test was able to detect most of the carbapenemase producers. This was also noted by another previous report (Pasteran *et al*., 2015).

CONCLUSION

We conclude that CP-CRK is a major health problem in the coming years. Hence, it is necessary to take all adequate measures to identify the resistant strains. Continuous monitoring of these resistant mechanisms is required to establish the changes in the prevalence and sensitivity pattern of MDR *Klebsiella* isolates. Urgent infection control measures coupled with antibiotic stewardship and strengthening of the healthcare infrastructure are to be instituted in our setting to prevent the spread of these carbapenem-resistant genes of *Klebsiella pneumoniae* (KPC).

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