Molecular Study of KPC-Mediated Carbapenem Resistance in *Klebsiella pneumoniae*

Running title: Molecular study of KPC-mediated carbapenem resistance

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Abstract

Background: The global distribution of *Klebsiella pneumoniae* that producescarbapenemase has gradually increased.This study investigated the molecular characterization of carbapenem-resistant *Klebsiella pneumoniae* isolates from various clinical samples.

Methods: This cross-sectional study was conducted in the Department of Microbiology at Sree Gokulam Medical College and Research Foundation in Venjaramoodu, Trivandrum, Kerala, India from January 2020 to January 2021. Various clinical samples were collectedto determine bacterial strains of *Klebsiella* species. The identification and antibiotic susceptibility testing of isolates were performedusingthe Vitek system. All the carbapenemase-producing carbapenem-resistant *Klebsiella pneumoniae* isolates (CP-CRK) were subjected to a multiplex polymerase chain reaction (PCR), to identify Class A β-lactamases producers (KPC), Class B β-lactamases producers (NDM), and Class D β-lactamases producers (OXA-48).

Result:401 clinical isolates of *Klebsiella* species were obtained from various clinical samples. The isolates were identified as *Klebsiella pneumoniae* (N=390; 97%) and *Klebsiella oxytoca* (N=11; 3%). 47 out of 401 (11.7%)isolates were multidrug-resistant and carbapenemase producers.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%). 36 (8.9%) *Klebsiella* isolates were positive for carbapenemase production.The overall prevalence of KPC, NDM, and OXA-48 in the CP-CRK was 21.3%, 2.1%, and 2.1% respectively.

Conclusion: The present study concluded that *Klebsiella pneumoniae*carbapenemase is the most common carbapenemase. The study also demonstrated that higher fatality rates were seen in the patients who had previously received colistin and carbapenem therapy.

Keywords: Carbapenem resistance, *Klebsiellapneumoniae*, polymerase chain reaction, Modified Hodge test, Carba NP test

Introduction

In the natural world, many *Klebsiella* species can be found. They are to blame for a lot of infections that occur in hospitals and other places. It belongs to the gram-negative bacteria family, which is also the cause of hospital-acquired illnesses. They might cause isolated infections or pandemics. Recent research has shown that the antibiotic class known as carbapenems is becoming less effective against the *Klebsiella* bacterium. Doctors typically utilize broad-spectrum carbapenems as a last resort for efficient treatment (1). Antibiotic resistance has significantly developed in *Klebsiella* species, and it is associated with significant mortality and morbidity (2).

A significant problem is the spread of carbapenem-resistant *Klebsiella* species worldwide (3). Carbapenemases are the major mechanisms of resistance, and carbapenemase-harboring isolates have been reported from across the globe with varying rates of prevalence. Globally, *Klebsiella pneumoniae*(*K.pneumonia*) carbapenemase (KPC) is the most common carbapenemase, while in the Indian subcontinent, New Delhi metallo-β-lactamase (NDM), which supposedly originated in India in 2008, is the most commonly reported carbapenemase (4, 5). Various variants of NDM differing from each other in one or a few nucleotides have emerged over the years (6, 7). Besides NDM, oxacillinase-48 (OXA-48)-like enzymes have also been reported from India (8). In India, NDM has spread to community and has been reported from public tap water and sewage isolates (9). Determine carbapenemase-producing, carbapenem-resistant *Klebsiella* as soon as feasible to prevent spread in a hospital environment (10). There is a serious issue with clinical practise and public health due to the increase of carbapenem resistance in Klebsiella species (11).

In *Klebsiella* species, increased carbapenem synthesis and reduced extracellular protein production are frequent causes of carbapenem resistance (12). Treatment choices are unaffected by the sort of carbapenem resistance mechanism present in this case. Currently, treatment considerations do not necessitate understanding the mechanism of carbapenem resistance. As a result, the practice is rare in diagnostic laboratories. The emergence of carbapenem-resistant *Klebsiella* species (CRK) has become a substantial therapeutic issue as a result of the lack of efficient treatments. In light of the CRK findings, a complete infection control effort must be performed (13).

Diagnostic laboratories are not advised by the Centers for Disease Control and Prevention (CDC) to manufacture carbapenemase (14). Several results have also been proposed by the Clinical and Laboratory Standards Institute (CLSI) to monitor carbapenemase production. Standard microbiology laboratories can only detect molecular detection methods in resource-rich environments. To ascertain the prevalence percentage rate of CRKas well as its morphological and genotypic characteristics in patients from a Tertiary Care Hospital in Kerala, this study was carried out.

Methods

This cross-sectional study was conducted in the Department of Microbiology at SreeGokulam Medical College and Research Foundation in Venjaramoodu, Trivandrum, Kerala, India from January 2020 to January 2021. The various clinical samples including blood, urine, pus, sputum, wound swab, and other body fluids received in the microbiology laboratory for *Klebsiella* isolation.

The isolates were identified with standard microbiological procedures.Standard bacteriological techniques were used for the isolation of the organisms (15). The VITEK 2 automated system (Biomerieux, France; with the GN and AST-N405 cards) was also used for the identification of isolates and antibiotic susceptibility testing. Isolates confirmed as *K.pneumoniae* and showing resistance to carbapenems based on minimum inhibitory concentration (MIC) values (as

determined by the Vitek system and interpreted according to CLSI guidelines) were further subjected to a polymerase chain reaction (Qiagen Rotor Gene Rt PCR) for determining the presence of various carbapenemase genes. According to the CLSI recommendation, the production of carbapenemases producing carbapenem-resistant *Klebsiella*is detected usingmodified Hodge and Carba-NP tests.The Quality Control strains used inmodified Hodge and Carba-NPtestsarecarbapenemase positive – *K.pneumoniae* ATCC BAA- 1705 and Carbapenemase negative- *K.pneumoniae* ATCC BAA – 1706.

DNA Extraction: The isolates to be tested were inoculated into a 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 and the pellet was used for DNA extraction.The pellet was suspended in 200µl of phosphate-buffered saline and 50µl of lysozyme was added and incubated at 37°C for 15min. Then 400µl of lysis buffer and 40µl of proteinase K (10mg/ml) were added and gently mixed well. This was incubated in a water bath at 70°C for 10 min. The whole lysate was transferred into the Pure Fast spin column and centrifuged at 10000 rpm for 1min. The flow through was discarded and 500µl of wash buffer was added and centrifuged at 10000 rpm for 1 min. The wash procedure was repeated one more time. The flow-through was discarded and the column was centrifuged for an additional 2 minutes to remove any residual ethanol. DNA was eluted by adding 100µl of elution buffer and it was centrifuged for 1min. 1µl of extracted DNA was used for PCR amplification.

The Polymerase Chain Reaction 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 Mgcl2, 0.4mM dNTPs mix, and 2U Proofreading Taq DNA polymerase. The primers used were

Forward Primer: 5'-GCT CAG GCG CAA CTG TAA G-3'

Reverse Primer: 5'-AGC ACA GCG GCA GCA AGA AAG-3'

The PCR vial was placed in a PCR machine and it was subjected to initial denaturation at 94°C for 3 min, 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 min. Gel electrophoresis was then carried out using 2% agarose gel. Gel was viewed in a UV transilluminator and the band pattern was observed.

Ethical approval was obtained from the Sree Gokulam Medical College and Research Foundation, Ethics Committee (Approval No.46/670/07/2022F). Written informed consent was obtained from all participants prior to their inclusion in the study.

Microsoft Office Excel 2010 was used to handle and prepare the data. Data were analyzed using SPSS v21 software. The data are represented as absolute numbers and percentages. All major data analyses were performed using Microsoft Excel. A $p < 0.05$ was taken for all statistical tests to indicate a significant difference.

Results

During the study span, 401 clinical isolates of *Klebsiella* species were obtained from various clinical samples including blood (N=95), urine (N=115), pus (N=96), sputum (N=71), wound swab (N=15), and other body fluids (N=9). The isolates were identified as *Klebsiellapneumoniae*(N=390; 97%) and *Klebsiellaoxytoca* (N=11; 3%).

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%).

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 was confirmed by the Modified Hodge test and Carba NP test. The results of the phenotypic characterization was shown in Table 1.

Table 1: Phenotypic Characterization of *Klebsiella* spp.

Isolate	**Number (Percentage)**
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%)

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.

Distribution of carbapenemases among various clinical samples

Distribution patterns of carbapenemase producers among 12 isolates were examined and shown in Table 2.

Table 2: Distribution of Carbapenemase producers among various samples

Sample	Carbapenemaseproducers No. (percentage)
Pus	6(50)
Urine	3(25)
Sputum	2(17)
Blood	1(8)

Among the 12 patients infected with CRK were retrospectively reviewed (Table 3). The patient's age, gender, underlying illnesses, and antibiotic use were noted for study analysis.An element of the clinical profile that was observed to increase the risk of infection by CRK was prior antibiotic use. Before the isolation of CRK infections, all patients received various antimicrobial treatments (e.g., carbapenem, piperacillin-tazobactam, colistin, third or fourth-generation cephalosporins, and/or fluoroquinolones).

Table 3: Bivariate analysis of demographic and clinical characteristics of patients with infections caused by Carbapenem Resistant *Klebsiellapneumoniae*(n=12)

Characteristics	Non survivors (died)	Survivors (Discharged from the hospital)	P
Age (25-95)	9 (75%)	3 (25%)	0.905
Genger			
Male	5 (42%)	1(8%)	0.459
Female	4 (33%)	2 (17%)	0.462
Comorbidities > 2	9 (75%)	3 (25%)	0.235
Risk factors			
Ventilator associated pneumonia	5 (42%)	2 (17%)	0.379
Chronic obstructive pulmonary disease	8 (67%)	2 (17%)	0.392
Kidney failure	2 (17%)	1(8%)	0.040
Diabetes	8 (67%)	1(8%)	0.001
HIV	1(8%)	0	0.718
Hepatitis	3 (25%)	2 (17%)	0.498
Cancer	2 (17%)	0	0.268
Heart failure	3 (25%)	1(8%)	0.281
Stroke	1(8%)	1(8%)	0.607
Septicaemia	2 (17%)	1(8%)	0.268
UTI	8 (67%)	2 (17%)	0.287
Meningitis	3 (25%)	0	0.281
Antibiotic therapy during the last month before carbapenem resistant *K. pneumoniae* isolation			
Colistin	10 (83%)	2 (17%)	0.030
Carbapenems	9 (75%)	2 (17%)	0.001
Third-generation cephalosporins	8 (67%)	2 (17%)	0.459
Fourth-generation cephalosporins	8 (67%)	1(8%)	0.346
Piperacillin-tazobactam	7 (58%)	2 (17%)	0.424
Tigecycline	8 (67%)	1(8%)	0.412
Aminoglycosides	6 (50%)	1(8%)	0.356

All the carbapenemase-producing isolates were found to be resistant to three or more classes of antibiotics and are 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 cotrimoxazole (83%). Overall antibiogram pattern of the isolates showed a high degree of sensitivity toward colistin (94%).

All the carbapenemase-producing carbapenem-resistant *Klebsiellapneumoniae* isolates (CP-CRK) were subjected to a multiplex polymerase chain reaction (PCR),to identifyClass A β-lactamases producers (KPC),Class B β-lactamases producers (NDM), and Class D β-lactamases producers (OXA-48). It was noted that 10 isolates expressed KPC followed by one isolate expressed NDM

and one isolate expressed OXA-48 (Table 4).The overall prevalence of KPC, NDM, and OXA-48 in the CP-CRK was 21.3%, 2.1%, and 2.1% respectively.

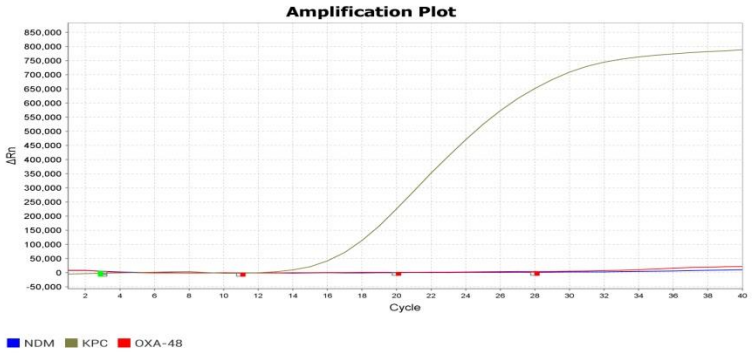

Figure 2: Show the results of the Real-Time PCR results for KPC in Klebsiella

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

Isolate	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

Discussion

Enterobacteriaceae, which makes up a sizable portion of the bacteria in the human gut and is capable of developing carbapenem resistance, includes *Klebsiella* species (16). Since carbapenems are the last choice in the treatment of life-threatening infections brought on by drug-resistant Enterobacteriaceae, the emergence and spread of carbapenem resistance among *Klebsiella* species represent a significant threat to public health (17).

In the present study,mostof the isolates were identified as *K.pneumoniae*. This finding is comparable to the reports of (18). and Sridhar Rao et al 2015. *K.pneumoniae* was the predominant species isolated in all the previous reports including the present study.

In the present study, the isolation rate of *Klebsiella* spp. is more from pus (44.7%) followed by urine (32 %), sputum (17%), and blood (6.3%). (19) reported that pus samples were the major source of *Klebsiella* spp. infection followed by sputum samples. According to (20), *K. pneumoniae* isolated from pus, sputum, and urine samples is also resistant to amoxiclav and ofloxacin.

In this study 11.72% (47/401) *Klebsiella* spp. was multidrug-resistant. This study was also supported by (21) 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 *Klebsiellapneumoniae* were 3%. This was also supported by (22) that 14.65% of the *K. pneumoniae* strains were resistant to carbapenems.

Of the 401 Klebsiella isolates, 12 (3%) were positive for carbapenemase production in the screening test and confirmation 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 (23) reported the higher prevalence (82.6%) of Modified Hodge test positive *K. pneumoniae* in India.

All Carbapenemase-producing isolates were discovered to be multidrug-resistant to at least three classes of antibiotics. Ceftazidime, cefepime, ciprofloxacin, tetracycline, piperacillin/tazobactam, imipenem, and meropenem were all completely resistant to all carbapenemase positive isolates. Gentamicin (75%), amikacin (70%), and cotrimoxazole (83%) all showed high resistance. Colistin had a 94% success rate in killing *K. pneumoniae*. So, for isolates of *K. pneumoniae* that are resistant to carbapenem, colistin might be the preferred medication. (24) exhibited great sensitivity against colistin. More isolates in this investigation (11.72%) were MDR. Many studies have supported that increase MDR is due to the easy availability and blind irrational use of antibiotics without proper culture report and prescription (25).

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 (26).

This study demonstrated that, higher fatality rates were seen in the patients who had previously received colistin and carbapenem therapy. Infections with these organisms have been linked to high rates of morbidity and mortality. This was also supported by (27) that a larger proportion of deaths were associated with CRK.

In this study, 11 isolates of *K. pneumoniae* was negative for all the tested carbapenemases. This indicates that these isolates of *K. pneumoniae* has mechanisms other than carbapenemase-like efflux pumps or porins to resist the carbapenems, which need further investigation. This baseline data generated from this study in a multispeciality hospital is important in view of the growing antibiotic resistance.

Conclusion

We conclude that CP-CRK will pose a serious threat to public health in the years to come, so it is imperative to take all necessary steps to find resistant strains. This study demonstrated that higher fatality rates were seen in the patients who had previously received colistin and carbapenem therapy. Infections with these organisms have been linked to high rates of morbidity and mortality. The present study concluded that KPC is the most common carbapenemase. It is a major health problem in the coming years and hence it is necessary to take adequate measures to identify the resistant strains. The presence of plasmid-based KPC calls for stricter surveillance measures in our hospital settings.

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Ethical statement

Ethical approval was obtained from the Sree Gokulam Medical College and Research Foundation, Ethics Committee (Approval No.46/670/07/2022F). Written informed consent was obtained from all participants prior to their inclusion in the study.

Author contributions

First author developed the research idea and designed the study. Second author performed the data collection and statistical analysis. Third author collaborated on writing and editing the manuscript. All authors read and approved the final version of the manuscript.

Conflict of interest

The authors declare no conflict of interests.

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