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Full Length Article

Prevalence and antimicrobial mechanism of carbapenem-resistant *Klebsiella pneumoniae* and its molecular properties

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ABSTRACT

Background: The carbapenem-resistant *Klebsiella pneumoniae* poses a serious threat to public health because carbapenems are used as a final resort to treat *K. pneumoniae*-mediated infections in humans.
Methods: Samples were collected from various clinical specimens including skin swabs, anal swabs, wound swabs, oral swabs, and sputum by the standard method and *K. pneumoniae* strains were isolated. Biofilm-forming characters were determined. The antibiotic resistance pattern was analyzed by the Kirby–Bauer disk diffusion method. Carbapenem resistance properties were tested using the imipenem and meropenem antibiotics. The carbapenemase genes (bla_{KPC} and bla_{NDM}) were determined.
Results: A total of 11 cephalosporin-resistant *K. pneumoniae* (CRKP) strains were isolated from the samples. The screened CRKP strains exhibited multi-drug resistance and non-susceptible to imipenem, ceftazidime, piperacillin, ceftriaxone, cefazolin, ampicillin, aztreonam, and cefotetan antibiotics. A total of 79.4 % K. pneumoniae isolates showed positive results on String-forming test. About 82.1 % of isolates showed mucoid colonies and 59 % of *K. pneumoniae* strains formed biofilm ($p < 0.01$). Out of 11 isolates, four strains exhibited *Klebsiella pneumoniae* carbapenemase type, three strains produced metallo-β-lactamases (MBL), and four strains exhibited as carbapenemase types. A total of 63.5 % *Klebsiella pneumoniae* carbapenemase-producing strains showed very low MIC value (<0.05 mg/L).
Conclusions: Drug-resistance K. pneumoniae was isolated from clinical specimens that were screened. The continuous monitoring of drug-resistance genes are required to make policy decisions.

1. Introduction

Antibiotic resistance is one of the major healthcare issues worldwide. The increasing interest towards multidrug resistance, and exploring molecular mechanisms and analyzing resistance genes were increased rapidly (Rodriguez-Mozaz et al., 2015). The unrestricted application of commercial antibiotics is a major factor and associated with the increase in antimicrobial resistant microorganisms (Gottlieb and Nimmo, 2011). For the past few decades, antibiotics are not only applied in the healthcare sector but also in agriculture and veterinary. The increased amount of drug-resistant bacteria as well as antibiotic-resistance genes was prevalent in animal and human feces. Microbiota from human beings can be enriched and altered with antibiotic resistance bacteria, hence, humans can be considered as a reservoir of antibiotic resistance genes and bacteria (Rodriguez-Mozaz et al., 2015). Humans acquired antibiotic resistance through two different pathways: Community and Hospital-acquired. Moreover, antibiotic resistance bacteria are highly common in hospital and healthcare settings (Hocquet et al., 2016). Nosocomial bacteria termed "ESKAPE'' include both Gram-negative and Gram-positive characterized by various drug resistance patterns and are involved in life-threatening infections in humans (Santajit and Indrawattana, 2016). Antibiotic resistance is a major burden and resistant to

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final-line resort antimicrobials, such as glycopeptides, fluoroquinolones, and carbapenems, and cephalosporins (third-generation antimicrobials) posing the serious threat to humans (Mckenna, 2013). Antibiotic resistance against these types of drugs was determined in the hospital environment and is being identified in community-acquired infections. These include vancomycin-resistant *Klebsiella pneumoniae*, multidrug resistant *Acinetobacter baumannii* and *Escherichia coli* (Cassini et al., 2019). Moreover, carbapenem-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* and third-generation cephalosporin-resistant and carbapenem-resistant Enterobacteriaceae are considered critical-priority bacteria, whereas vancomycin resistant enterobaceriaceae, together with methicillin-resistant *Staphylococcus aureus*, has high priority based on WHO list of antibacterial resistant bacteria. The prevalence of *K. pneumoniae*, especially carbapenems-resistant strains is a global healthcare concern of various countries, including Africa, Asia and Europe. The beta-lactamases producing *K. pneumoniae* among Enterobacteriaceae have been already reported and the subtype of beta-lactamases was metallo-β-lactamases and class D β-lactamases (Findlay et al., 2017). The main aim of the present study was to analyze the prevalence of drug resistant carbapenemase-producing *K. pneumoniae* in the specimens using biochemical and molecular methods.

2. Materials and methods

2.1. Samples

The test strains were collected at a microbiology lab where they were isolated from a variety of clinical specimens, such as sputum, skin, anal, and wound swabs, as well as swabs from patients who had been admitted to the hospitals between March 2021 and February 2022.

2.2. Growth characteristics of K. pneumoniae, mucinous phenotype and biofilm formation

The mucinous phenotype of *K. pneumoniae* was evaluated as described previously (Yang et al., 2019). Briefly, the isolated *K. pneumoniae* strains were spread on Columbia blood agar plates and incubated for 24 h at 37°C. The colony was dipped in the inoculum ring and left the inoculum ring for 24 h. The development of colonies on the adhesive wire was analyzed and the adhesive formed >0.5 cm was considered as positive. The bacterial strains were cultured on MacConkey agar medium and incubated for 24 h at 37°C. After 24 h, the development of mucoid colonies was observed. The biofilm-forming potential was analyzed by evaluating the adhesion to the microtiter plate method. Briefly, the isolated bacterial strains (18 h) were inoculated into the 96-well plate containing nutrient broth medium. The microtiter plates were incubated for 48 h at 37°C and the well was washed with phosphate-buffered saline (three times) and dried at 50°C. The wells were stained with 100 µL of 1 % crystal violet for 10 min and washed the well to remove the excess stain. It was air-dried and the wells were flooded with 350 µL of 95 % ethanol and agitated for 10 min. The absorbance was read at 570 nm against the reagent blank. The colour intensity was measured and was classified as weak biofilm producers, moderate biofilm producers and very strong biofilm producers.

2.3. Antibiotic susceptibility analysis

The antibiotic resistance pattern was analyzed by the Kirby–Bauer disk diffusion method using Mueller–Hinton agar plates (Zhang et al., 2020). Carbapenem resistance properties of *K. pneumoniae* were evaluated using the imipenem and meropenem antibiotic disks. The antibiotics used were: amikacin, imipenem, ceftazidime, piperacillin, trimethoprim-sulfamethoxazole, ceftriaxone, ampicillin, levofloxacin, cefazolin, cefotetan. gentamicin, aztreonam and sulbactam. *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922) were used as the standard stains.

2.4. Detection of carbapenemases from drug resistant Klebsiella isolates

Meropenem and Imipenem resistant *Klebsiella* strains were used for carbapenemases screening using the modified Hodge test (MHT method), and the meropenem combined disk test (CDT method) as described previously. Briefly, the bacterial strains were cultured in MHB medium and it was diluted appropriately (1:10 dilution of 0.5 McFarland) *E. coli* suspension (ATCC 25922) was placed on MHA plates and meropenem was loaded at the centre of the plate. The selected *Klebsiella* strains were inoculated in a straight line (edge of the plate to the edge of the meropenem disk). A positive control (*K. pneumoniae* ATCC BAA-1705) was used to validate the results. The culture plates were incubated at 32°C for 24 h and the results were observed. CDT method was used for the determination of carbapenemase-producing enterobacteria as described earlier by Pournaras et al. (2013). The drug-resistant strains were inoculated on MHA plates. Then antibiotic discs meropenem with phenylboronic acid, and meropenem, meropenem with PBA, and meropenem with EDTA were placed onto the MHA plates. The plates were incubated for 24 h at 32°C and the result was compared with the control (Tsakris et al., 2010).

2.5. Extraction of DNA and analysis of carbapenemase genes (bla_{KPC} and bla_{NDM})

K. pneumoniae DNA was extracted using a DNA purification kit (Qiagen, Germany) as described earlier (El-Badawy et al., 2017). Briefly, the bacterial colonies were mixed in 200 µL of Millipore double distilled water in a 1.5 mL microcentrifuge tube. The culture was vortexed for a few seconds and kept in a boiling water bath at 94 °C for 8 min to lyse the selected culture. It was further centrifuged for 10 min at 10,000 rpm. The total genomic DNA was retained from the supernatant and was measured using a Nanodrop Spectrophotometer (Thermo Scientific, USA). Polymerase chain reaction was carried out to detect bla_{KPC} and bla_{NDM} carbapenemase genes by forward and reverse primers. The amplified PCR products were analyzed by 1.5 % agarose gel electrophoresis. The amplified gene product was further visualized under a UV *trans*-illuminator.

2.6. Antimicrobial activity of antibiotics against carbapenemase type bacterial strains

A microbroth dilution method was used for the determination of antimicrobial susceptibility. Briefly, the culture medium was sterilized and loaded into a microtiter plate and antimicrobial agents were added at various concentrations and combinations and incubated for 24 h at 28 ± 1 °C. After 24 h, the optical density was measured using a spectrophotometer (Wu et al., 2020). A total of fifteen antimicrobial agents (ampicillin–sulbactam, ampicillin, piperacillin–tazobactam, ceftazidime, cefepime, cefoperazone–sulbactam, amikacin, Gentamicin, Levofloxacin, and trimethoprim-sulfamethoxazol, Ceftazidime–avibactam, Meropenem–Vaborbaktam and Imipenem/cilastatin/relebactam) were used. To prevent the control of extended-spectrum beta-lactamase microbes, cephalosporin was selected at two different combinations (ceftazidime-clavulanate and cefotaxime-clavulanate). The MIC value was determined as described previously (Malar et al., 2020).

3. Results

3.1. Distribution of carbapenemase-producing Klebsiella pneumoniae

A total of 108 patients were subjected to the determination of carbapenemase-producing *K. pneumoniae*. In 108 patients, carbapenemase-producing *K. pneumoniae* (CRKP) strains were isolated and a total of 11 CRKP strains were obtained in this study. Two CRKP isolates were isolated from the skin, whereas seven were isolated from the wound and the remaining two were obtained from the sputum

collections ($p < 0.01$). A significant CRKP population was detected in the wound sample and no CRKP positive strains were detected in the anal and oral swabs (Fig. 1A). The analyzed age of CRKP-positive patients varied from 9 to 78 years. The old age group (76–100) represented 63.3 % of CRKP isolates cases and this proportion was significantly less (36.6 %) for the 26–75 age groups ($p < 0.01$) (Fig. 1B).

3.2. Antibiotic susceptibility testing

The drug resistance patterns of CRKP strains were illustrated in (Table 1). The isolated CRKP bacteria exhibited non-susceptible to imipenem, ceftazidime, piperacillin, ceftriaxone, cefazolin, ampicillin, aztreonam, and cefotetan. The CRKP strains exhibited intermediate or sensitivity to amikacin, trimethoprim-sulfamethoxazole, levofloxacin, gentamicin, and sulbactam. In our study, 68.3 % of the isolated strains exhibited multidrug resistance. The MIC values were determined against CRKP strains and the susceptibility patterns of isolates are presented in Table 1.

Table 1
Antimicrobial susceptibility of CRKP to antimicrobial agents.

Antibiotics	Antibiotic susceptibility (%)			MIC (μg/mL)		
	S	I	R	S	I	R
Imipenem	0	0	100	1	3	≥5
Ceftazidime	0	0	100	5	10	≥20
trimethoprim-sulfamethoxazole	56.5	0	43.5	4	8	≥25
Ampicillin	0	0	100	4	8	≥16
Piperacillin/tazobactam	0	0	100	25	75	≥125
Ceftriaxone	0	0	100	1	4	≥10
Amikacin	50	10	40	8	16	≥32
Cefazolin	0	0	100	8	16	≥32
Gentamicin	42	18	40	2	4	≥8
Ampicillin/Sulbactam	0	0	100	8	16	≥32
Aztreonam	0	0	100	2	4	≥8
Cefotetan	0	0	100	32	64	≥128
Levofloxacin	54.5	2.9	42.6	4	8	≥16
Meropenem	0	0	100	5	10	≥125

S – sensitive, I – intermediate, R – resistant.

3.3. Mucinous screening, mucoid colonies development and biofilm-forming characteristics of K. pneumoniae

A total of 79.4 % *K. pneumoniae* isolates showed positive results on the string-forming test. Most of the string-formed *K. pneumoniae* isolates exhibited multidrug resistance. In the present study, mucoid and pink colour colonies were observed on MacConkey agar plates. The non-mucoid colonies were also observed. About 82.1 % of isolates showed mucoid colonies and the remaining 17.9 % exhibited non-mucoid properties (Fig. 2A and B). A total of 11 *K. pneumoniae* isolates were tested for the analysis of biofilm production and these isolates were

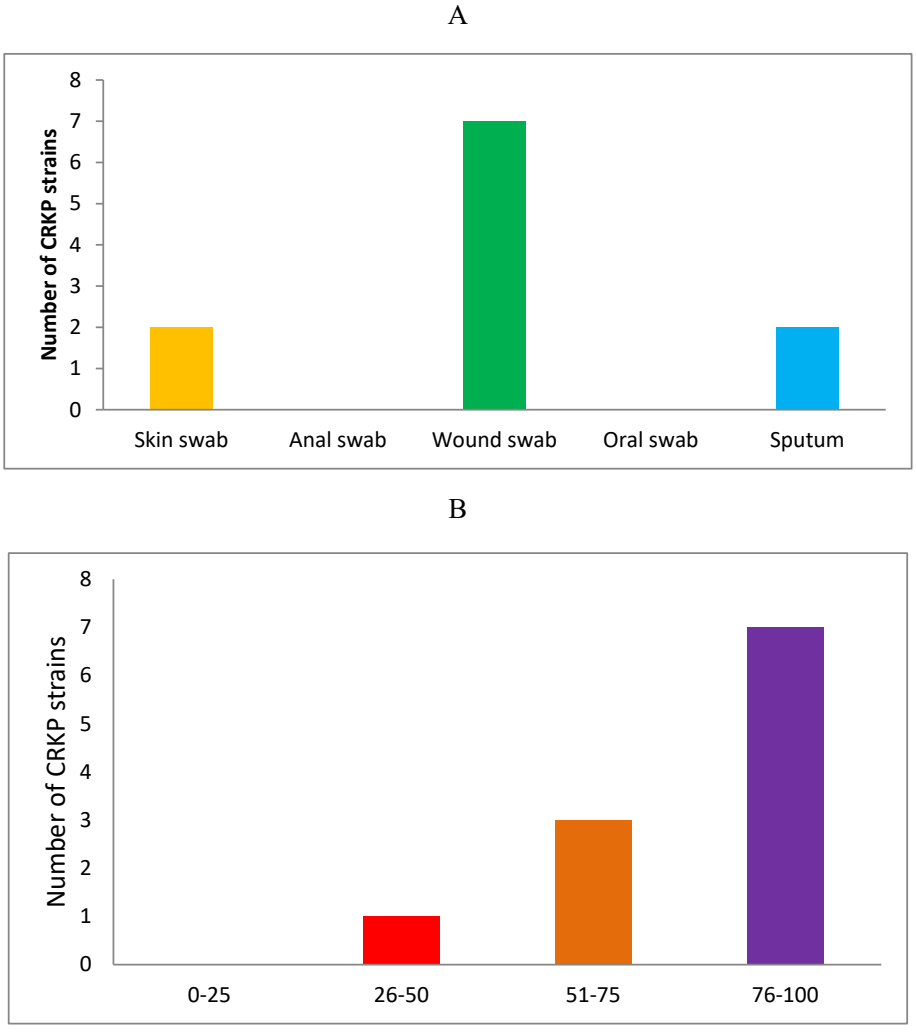

Fig. 1. Distribution of carbapenemase-producing *Klebsiella pneumoniae* from the patients admitted to the hospitals (A). Frequency of CRKP bacteria among different age groups (B).

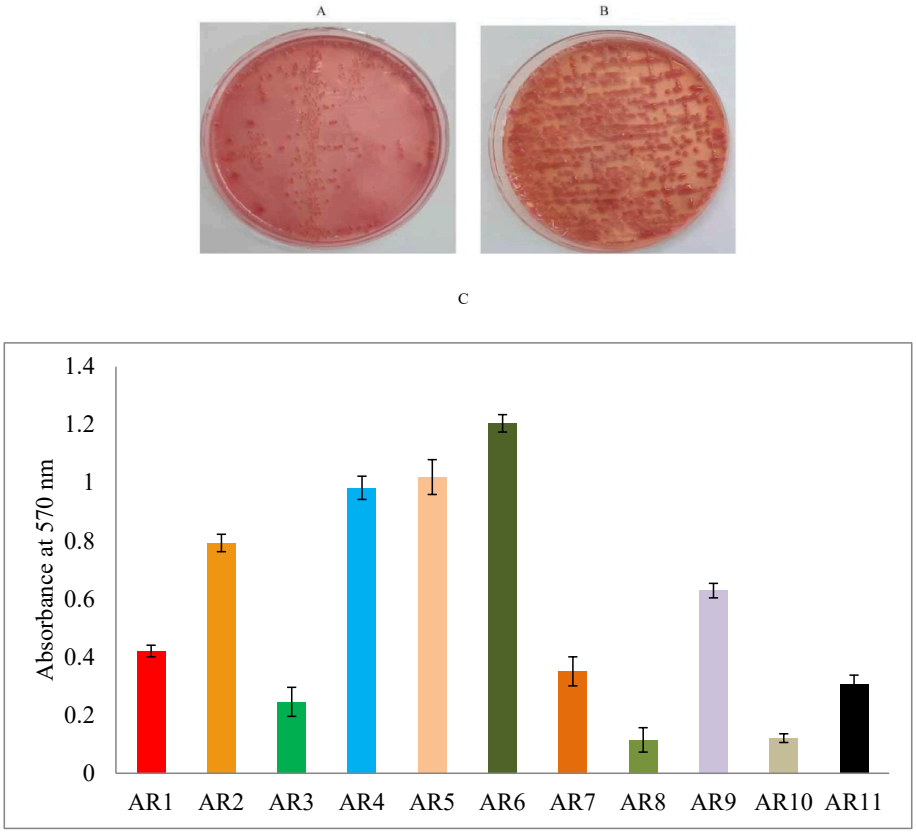

Fig. 2. Development of non-mucoid (A) and mucoid (A) K. pneumoniae colonies on MacConkey agar plates. The isolates were placed on MacConkey agar medium and incubated for 24 h. (C) The biofilm-producing potential of *K. pneumoniae* isolated from the clinical specimens.

CRKP types. Among multi-drug resistant bacterial strains, 59 % of *K. pneumoniae* strains presented biofilm-formation and the remaining isolates were non-multidrug resistant strains. The strains AR4, AR5, and AR6 exhibited maximum biofilm-production and the absorbance values were 0.983 ± 0.04, 1.02 ± 0.06, and 1.205 ± 0.03, respectively (Fig. 2C).

3.4. Carbapenemase-producing K. pneumoniae

Carbapenemase-producing bacterial strains were determined using Modified Hodge Test (MHT) and Combined disk test (CDT). MHT analysis revealed the presence of 9 carbapenemases-producing strains (63.6 %) and a combined disk test detected carbapenemases-producing strains. Out of 11 isolates, four strains exhibited KPC type, three strains produced MBL, and four strains showed KPC/MBL types. The variation in the result revealed sensitivity between the selected methods (Table 2).

Table 2
Determination of carbapenemase-producing bacterial strains by modified Hodge test and combined disk test.

*K. pneumoniae*strains	Modified Hodge Test	Combined disk test
AR1	+	KPC
AR2	−	KPC/MBL
AR3	+	MBL
AR4	+	KPC
AR5	+	KPC/MPL
AR6	+	KPC/MBL
AR7	−	MBL
AR8	+	KPC
AR9	+	MBL
AR10	+	KPC/MBL
AR11	+	KPC

+: positive; −: negative; KPC − *K. pneumoniae* carbapenemases; MBL – metallo-β-lactamases.

3.5. Detection of carbapenemase-producing genes (bla_{NDM} and bla_{KPC} genes) from K. pneumonia strains

The isolated 11 carbapenemase-producing *K. pneumoniae* strains were subjected to the determination of bla_{NDM} and bla_{KPC} genes. The bla_{NDM} was detected in four *K. pneumoniae*strains while bla_{KPC} gene was detected in 8 bacterial strains. Fig. 3 presented the amplified bla_{NDM} and bla_{KPC} genes on agarose gel electrophoresis. Of the 11 drug-resistant isolates, four bacteria exhibited both bla_{NDM} and bla_{KPC} genes. Statistical analysis revealed that there is a significant variation in the presence of drug resistance genes (bla_{NDM} *and* bla_{KPC}) among the *K. pneumoniae*

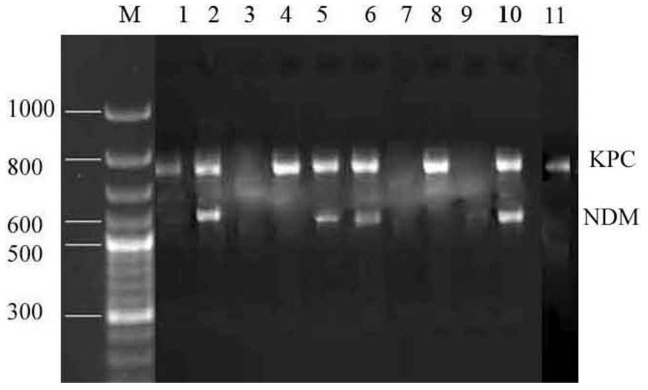

Fig. 3. Detection of the carbapenemase genes (KPC and NDM) in carbapenemase-synthesizing *K. pneumoniae* isolates 1 to 11. Lane M –: DNA ladder, Lane 1–11: *bla* KPC and *bla* NDM genes of *K. pneumoniae*.

strains ($p < 0.01$).

3.6. In vitro antibiotic activity against carbapenemase-producing carbapenem-resistant K. pneumoniae

In vitro antibiotic activity was tested against carbapenemase-producing carbapenem-resistant *K. pneumoniae*. Ceftazidime (non-β-lactam β-lactamase inhibitor) in combination with avibactam (non-β-lactam β-lactamase inhibitor) was tested against KPC-producing *K. pneumoniae* and all strains showed sensitivity. KPC-producing strain presented MIC ranged between 0.025 and 1 mg/L. The combination of Meropenem–Vaborbaktam was more effective than Ceftazidime-Vaborbaktam. *K. pneumoniae* was evaluated to test the susceptibility against cefiderocol (beta-lactamase inhibitor). The MIC value of cefiderocol was tested on *K. pneumoniae*, and KPC-producing strains showed lower MIC values than other tested antibiotics (see Table 3).

Table 3
In vitro antibiotics (Ceftazidime– avibactam, Meropenem– Vaborbaktam, and Cefiderocol) activity against carbapenemase-producing carbapenem-resistant *Klebsiella pneumoniae.*

Ceftazidime–avibactam MIC (mg/L)	KPC (n)	Meropenem–Vaborbaktam MIC (mg/L)	KPC (n)	Cefiderocol MIC (mg/L)	KPC (n)
0.025	1	0.025	1	0.0125	7
0.075	1	0.05	4	0.025	2
0.125	5	0.075	2	0.05	2
0.525	1	0.125	1	0.075	0
0.825	2	0.15	0	0.1	0
1	1	0.25	3	0.125	0

4. Discussion

K. pneumoniae is one of the opportunistic pathogenic bacteria that cause nonhospital infections and severe nosocomial infections. This pathogenic strain produces carbapenemases that have the potential to degrade β-lactam antibiotics, including various synthetic carbapenem antibiotics. The increasing emergence of β-lactam antibiotics cause increased mortality among hospitalized cases and carbapenems are considered as the antibiotics of final resort to treat bacterial infections from Gram-negative type (Politi et al., 2019). World Health Organization listed *K. pneumoniae* as a virulent organism (Aires-de-Sousa et al., 2019). These pathogenic strains were isolated from the skin, sputum, and wound samples. This organism was characterized with asymptomatic colonization on health care workers, patients, and it survives on various non-sterile medical equipment and apparatus (Moradigaravand et al., 2017). This facilitates for the simple transmission of carbapenem-hydrolyzing enzymes-coded genes from one plasmid to another. Moreover, several drug resistance mechanisms can be available on the same plasmid, which makes to the development of multidrug-resistant *K. pneumoniae* species. Aires-de-Sousa et al. (2019) isolated drug-resistant *K. pneumoniae* from the rectum of the patients and urine contributed 43.5 %, and 32.6 %% respectively. The present study revealed that the maximum number of *K. pneumonias* strains in a wound sample ($n = 7$) than other sources. The prevalence of *K. pneumoniae* varied based on the degree of infection and only 8.7 % of isolates were identified in the blood sample previously (Aires-de-Sousa et al., 2019). It has been previously reported that the carbapenemase-enzyme-producing *K. pneumoniae* strains exhibited antibiotic resistance against various drugs, including penicillins and cephalosporins (Gao et al., 2020). Durdu et al. (2019) reported that the isolated *K. pneumoniae* strains presented 91.3 % resistance to cefepime and 72.6 % resistance to ciprofloxacin. Moreover, the research work performed by Singh et al. (2017) reported 100 % resistant strain to cefepime and exhibited 70 % resistance to cefotaxime and 65 % resistance to ciprofloxacin. In our study, the selected *K. pneumoniae* showed 100 % resistance to imipenem, ceftazidime, piperacillin, ceftriaxone, cefazolin, ampicillin, aztreonam, and cefotetan. Apondi et al. (2016) reported that the isolated *K. pneumoniae* strains exhibited 82.8 % resistance to gentamicin and 21 % of bacteria showed resistance to amikacin. In the present study, 40 % of the isolated strains exhibited resistance against gentamicin and amikacin and the drug-resistant patterns are diverse among the type strains. The increased resistant *K. pneumoniae* strains were reported previously. A total of 99 % of strains exhibited resistance to meropenem and all the strains showed resistance to imipenem these findings revealed increased carbapenem resistance among bacterial strains (Zheng et al., 2017). The percentage of isolates resistant to meropenem and imipenem was 100 %. *K. pneumoniae* is one of the carbapenemases secreting bacteria and is classified as MBL (NDM), KPC and OXA-48 families (Yonekawa et al., 2020). It has been reported previously by WHO stated the prevalence of *K. pneumoniae* strains was more than 50 %. Recent epidemiological studies revealed that specific carbapenemases were predominant in various parts of the world. In Pakistan, India, and Sri Lanka and other Asian countries, NDM-producing *K. pneumoniae* were predominant. In the United States, Australia, China, Greece, South America, and Italy, NDM type carbapenemase type. Moreover, OXA-48 type carbapenemase-producing strains are endemic in North Africa, and the Middle East (Suay-García and Pérez-Gracia, 2021). In this study, the bla_{NDM} was detected in four K. pneumoniae strains while bla_{KPC} gene was detected in 8 bacterial strains. Of the 11 drug-resistant isolates, four bacteria exhibited both bla_{NDM} and bla_{KPC} genes. In China, KPC-producing strains contributed to >50 % *K. pneumoniae* population and NDM-type carbapenemases producing strains contributed to 11 % population and 37 % of strains were OXA-48 type carbapenemases (Han et al., 2020).

5. Conclusions

The present finding revealed the emergence of multidrug-resistant carbapenemase-producing *K. pneumoniae* in hospitalized patients. The tested bacterial strains exhibited resistance to multiple key antibiotics such as imipenem, ceftazidime, piperacillin, ceftriaxone, cefazolin, ampicillin, aztreonam, and cefotetan, which are regarded as serious threat to public health. The most common gene identified was *bla*KPC, while the *bla*NDM gene was less frequent.

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CRediT authorship contribution statement

Palanisamy Manikandan: Methodology, Investigation, Validation. **Saleh Aloyuni:** Conceptualization; Resources. **Ayoub Al Othaim:** Investigation, Funding acquisition, Validation, Writing – original draft. **Ahmed Ismail:** Conceptualization. **Alaguraj Veluchamy:** Writing – original draft, Writing – review & editing. **Bader Alshehri:** Writing – original draft, Writing – review & editing. **Ahmed Abdelhadi:** Validation, Methodology, Funding acquisition. **Rajendran Vijayakumar:** Methodology, Investigation, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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