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Emergence of extended spectrum beta-lactamases and carbapenemases producing *Klebsiella* spp. isolated from Hospital Sultan Abdul Aziz Shah

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Abstract. The emergence of extended spectrum β-lactamases (ESBLs) and carbapenemases producing *Klebsiella* spp. in hospital settings is alarming as it leads to resistance to carbapenems, the last resort of antibiotics. Therefore, the objective of this study is to determine the antibiogram profile and identify the resistance genes present in *Klebsiella* spp. clinical isolates, encompassing specimens such as urine, sputum, blood and pus. The presence of β-lactamase genes; *bla*TEM-1 and *bla*CTX-M-1 and carbapenemases genes; *bla*NDM-1 and *bla*VIM- 1 were detected by polymerase chain reaction (PCR) analysis. A total of 85 isolates were collected from ill patients at Hospital Sultan Abdul Aziz Shah (HSAAS) between January 2022 and March 2023. The resistance profile of these isolates was analysed using the Kirby-Bauer disk diffusion method against various classes of antibiotics. The results revealed that all 85 *Klebsiella* spp. tested were resistant to ampicillin and ciprofloxacin. Furthermore, 78% of isolates were resistant to cephalosporins, suggesting that these strains were producing ESBLs. In terms of carbapenem resistance, the isolates were more resistant towards meropenem (80%) than imipenem (60%). These 85 clinical isolates were also resistant to kanamycin (52%). The presence of *bla*TEM-1 and *bla*CTX-M-1 genes were detected in the tested isolates, namely strain number 15,18,27,64 and 65. Moreover, the carbapenem-non-susceptible isolates were detected to have *bla*NDM-1 and *bla*VIM-1 genes. These findings highlighted a substantial proportion of isolates as ESBLs and carbapenemase producers. In conclusion, this study emphasised the urgent need for enhanced surveillance programs to combat the escalating threats of multidrug resistance *Klebsiella* spp. in clinical settings. Additionally, routine molecular screening for resistance genes along with rapid diagnostics for detecting these genes, should be implemented to enable doctors to prescribe the correct antibiotic.

Keywords: *bla*NDM-1, *bla*VIM-1, *bla*TEM-1, *bla*CTX-M-*1*, ESBLs, *Klebsiella* spp.

INTRODUCTION

Klebsiella spp. is a group of Gram-negative bacteria belonging to the *Enterobacteriaceae* family. They are widely spread in the environment and commonly found in the gastrointestinal tract of animals and humans. In recent years, several strains of *Klebsiella* spp. have caused distress because of their ability to cause a variety of illnesses, such as surgical site infections, bloodstream infections, pneumoniae and urinary tract infections (Sharma *et al.,* 2023). The occurrence of multidrug-resistant (MDR) *Klebsiella* spp. has increased alarmingly in recent years. This is due to the production of

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extended-spectrum β-lactamases (ESBLs) and carbapenemases, along with the upregulation of efflux pump and changes in membrane permeability (Van Duin *et al.,* 2017). MDR *Klebsiella* spp. infections create challenges in medical facilities since they are linked with high rates of healthcare costs, illness, and deaths.

ESBLs are enzymes from Ambler molecular class A that make bacteria resistant to oxyimino-β-lactams, including third and fourth generation cephalosporins (e.g., cefotaxime, ceftazidime, ceftriaxone, cefixime, and cefepime), extended-spectrum penicillin such as piperacillin, and monobactams like aztreonam. These enzymes can be blocked by clavulanate and tazobactam. ESBLs fall under group 2be in the Bush-Jacoby-Medeiros classification system based on their molecular and biochemical functions. ESBLs can be encoded on plasmids, which are easily transferable between bacteria, facilitating the spread of these resistance genes and consequently leading to an increase in antimicrobial resistance (Bush, 2018).

The major ESBL families include TEM, SHV, CTX-M and OXA, each of which causes resistance to different classes of antibiotics, contributing to complications in the treatment of infections caused by *Klebsiella* spp. TEM and SHV-type β-lactamases were among the earliest identified, whereby TEM enzymes evolved from the initial TEM- 1 found in *E. coli* to hydrolyse extended-spectrum cephalosporins due to mutations. Similarly, SHV-type enzymes, derived from *Serratia marcescens*, hydrolysed extended-spectrum cephalosporins like cefotaxime and ceftazidime (Munoz-Price, 2022). CTX-M β-lactamases are concerning particularly in *Klebsiella pneumoniae,* originating from *Kluyvera* spp. because they confer high levels of resistance to cefotaxime and other third generation cephalosporins in clinical settings (Ortiz-Diez *et al.,* 2023). The OXA β-lactamase represent a growing family of ESBLs, differing from TEM and SHV enzymes as they belong to molecular class D and functional group 2d. These β-lactamases confer resistance to ampicillin and cephalothin, characterized by their ability to hydrolyse oxacillin and cloxacillin and poorly inhibited by clavulanic acid. Originally, the OXA-type enzymes were found in *Pseudomonas aeruginosa* (Bush *et al.,* 1995). These ESBLs are critically important for *Klebsiella* spp. due to their significant role in antibiotic resistance, which complicates the treatment of infections caused by these bacteria. *K. pneumoniae* and other *Klebsiella* species have increasingly shown resistance to commonly prescribed β-lactam antibiotics, including third generation cephalosporins (Priyanka *et al.,* 2020).

ESBL producing bacteria, which includes *K. pneumoniae*, pose challenges in hospitalized patients by increasing the rate of morbidity and mortality. A study conducted at a tertiary hospital located on the Eastern Coast of Malaysia between January 2018 and June 2020 revealed that *K. pneumoniae* (53.6%) and *Escherichia coli* (40.9%) were the most prevalent ESBL producers, with bacteremia as the most common diagnosis (42.7%). Despite maintaining a high level of susceptibility to carbapenems (≥99%), most isolates were resistant to penicillin and cephalosporins, highlighting heavy reliance on carbapenems for treatment. However, the hospital's mortality rate was 13.6%, with higher risks for patients in the ICU and those with diabetes mellitus. This situation emphasises the need for careful use of antibiotics to prevent the development of resistance (Abubakar *et al*., 2022).

Carbapenem is believed to be the last line of defence against diseases caused by Gram-negative bacteria which include *Klebsiella* spp. The emergence of carbapenem resistance in *Klebsiella* spp. is an increasingly serious clinical issue especially among individuals with weaker immune systems and those suffering from numerous illnesses. The growing concern on antibiotic resistance is further worsened by the rise of carbapenem-resistant *Enterobacteriaceae* (CRE), first reported by Rasmussen *et al.* in 1996. These multidrug resistant organisms have emerged as significant causes of infections, especially among immunocompromised patients. A study at 500-bed tertiary hospital in Selangor, Malaysia found that the prevalence of CRE rose from 0.3% in 2015 to 1.2% in 2016, with *K. pneumoniae* accounting for 81.8% of the isolates (Mohamed *et al*., 2018). Carbapenem resistance in *Klebsiella* spp. is primarily driven by carbapenemase enzymes, which belong to different classes based on their molecular structure and mechanisms of action. The main classes include Ambler class A (serine carbapenemase such as KPC), class B (metallo-β-lactamases like New Delhi metallo-β-lactamase-1

(NDM-1) and Verona integron- encoded metallo-β-lactamase (VIM-1)), and class D (OXA-type carbapenemases). Metallo-β-lactamases (class B), such as NDM-1 and VIM-1, can hydrolyze and inactivate a broad range of β-lactam antibiotics, including carbapenems (e.g., imipenem and meropenem), making them highly resistant to these antibiotics. Their rapid spread among bacteria, including *Klebsiella* spp. has become a major concern in antimicrobial resistance (Nordmann *et al.,* 2011). The presence of NDM-1 and VIM-1 genes in *Klebsiella* spp. strains may limit the treatment options and lead to increased morbidity, mortality and healthcare costs (Thapa *et al.,* 2021). The resistance towards the widely used carbapenems (imipenem and meropenem) can be mediated by several mechanisms which encompasses β-lactamases, alterations in porin, and modifications to penicillin-binding proteins (Rice, 2008). The most frequently detected carbapenemase genes were NDM-1 and OXA-48, either alone or in combination, which together made up 41% of the case. Alarmingly, all CRE isolates demonstrated resistance to cephalosporins and carbapenems, while high rates of resistance to tigecycline (46%), polymyxin B (17%), and colistin (17%) further limiting treatment options (Lau *et al*., 2021).

While extensive studies have highlighted the global threat of antimicrobial resistance there is a lack of data on the molecular epidemiology and resistance mechanisms of *Klebsiella* spp. isolates from specific regions, including Malaysia (Hidalgo-Tenorio *et al*., 2024; WHO, 2023). Additionally, the emergence of new β-lactamase variants and carbapenemase-producing strains poses a significant challenge to current treatment protocols, emphasizing the need for localized surveillance and molecular characterization of these resistant organisms (Bonomo, 2020). Surveillance and data sharing are vital aspects of a One Health approach. Establishing effective systems to track antibiotic-resistant pathogens across humans, animals, and the environment is essential. Encouraging collaboration and data exchange between health agencies, environmental groups, and research institutions is critical for understanding resistance trends comprehensively. Moreover, research and innovation are central to tackling antibiotic resistance. A One Health strategy promotes the development of new antibiotics, alternative treatments, and vaccines, all of which can reduce reliance on existing antibiotics (Muteeb *et al.,*2023). This study aims to fill the knowledge gap by investigating the antibiotic resistance and molecular epidemiology of *Klebsiella* spp. producing ESBLs and CRE from patient samples collected from common infections encountered in hospitalized patients at Hospital Sultan Abdul Aziz Shah (HSAAS). The study seeks to provide data that can support evidence-based practices and interventions for improved patient care and infection control strategies to combat the escalating challenge of MDR bacterial strains worldwide.

MATERIALS AND METHODS

Sample collection

A total of 85 clinical isolates of *Klebsiella* spp. were collected from different patients admitted to Hospital Sultan Abdul Aziz Shah (HSAAS), Selangor, Malaysia, between January 2022 to March 2023. The *Klebsiella* spp. isolates were acquired from different clinical specimens, including urine, sputum, blood, wound, rectal swab, tracheal aspirate, pus, bronchoalveolar lavage (BAL), and tissue. Bacterial isolates on agar plates were placed in an icebox immediately after collection and transported to the laboratory at Universiti Putra Malaysia (UPM) within 30 minutes to maintain viability. Upon arrival, the isolates were subcultured immediately onto fresh Luria Bertani (LB) agar plates and incubated overnight at 37°C. The following day, single colonies were inoculated into LB broth for bacterial growth and subsequent disk diffusion analysis. Remaining cultures were stored in 40% glycerol stocks at -80°C for further molecular and phenotypic analyses. Storage conditions were monitored to preserve bacterial viability and genetic integrity for future experiments. All the clinically isolated samples were identified as *Klebsiella* spp. by the hospital personnel using API test strips (Biomérieux, Marcy L'Etoile, France), following the manufacturer's guidelines (Pincus,2006). Followed by the IMViC biochemical test as described in the Clinical Microbiology Procedures Handbook (Leber,

2020) and in accordance with the M100 guidelines (CLSI, 2020).

Antibiotic susceptibility testing

The antibiogram profile of all clinical isolates was obtained by performing the Kirby-Bauer disk diffusion assay according to CLSI M02 performance standards for antimicrobial disk susceptibility tests (CLSI, 2018). The antibiotic disks (Oxoid Ltd., England) used are ceftazidime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), ampicillin (10 µg), imipenem (10 µg), meropenem (10 µg), ciprofloxacin (5 µg), norfloxacin (10 µg), chloramphenicol (30 µg), and kanamycin (30 µg). Bacterial isolates were individually prepared at a concentration of 0.5 McFarland using phosphate-buffered saline (PBS) and spread on the Mueller-Hinton agar (Merck, Germany) surface with a sterile swab. After 15 minutes, antibiotic discs were positioned on the surface of the media with 20mm distance from each other. The plates were incubated for 16 to 18 hours at 37°C. The diameter of the inhibitory zone was measured in millimeters (mm) and interpreted as susceptible, intermediate, or resistant, using the CLSI M100 guideline breakpoints for interpreting disk diffusion results (CLSI, 2020)

Molecular analysis of resistance-related genes

A subset of the phenotypically confirmed ESBLs, which are resistant to multiple antibiotics, especially cephalosporins, ampicillin, and ciprofloxacin, as well as strains resistant to imipenem and meropenem, were selected for further identification using PCR. Six strains with diverse antibiotic susceptibility profiles were tested for the presence of ESBL genes *bla*TEM-1 and *bla*CTX-M-1 group, and the carbapenemase genes, *bla*VIM-1 and *bla*NDM-1. Polymerase chain reactions (PCR) were performed using the Eppendorf Mastercycler® nexus gradient thermal cycler (Germany). The reverse and forward primers with the PCR conditions used for analyses are given in Table 1. The DNA amplification program consisted of an initial denaturation step at 94°C for 10 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 52°C for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes. PCR products were subjected to electrophoresis on a 1% agarose gel for 30 minutes at 100 V. It was then visualized, and photographed under UV illumination either with 100-bp DNA ladder from Generuler™ (Thermo Fisher Scientific U.S.A.) or 1kb DNA ladder (Exact Mark, 1st BASE Pte Ltd); as a molecular weight marker.

Table 1. Primers used for PCR amplification of ESBL and carbapenemase genes in *Klebsiella* spp. "F-" and "R-" denote forward and reverse primers, respectively. The table includes primer sequences, references, and the expected product sizes.

No.	Primer	Sequence (5'-3')	Reference	Product size (base pair)
1	*bla*VIM – 1	F – 5' GATGGTGTTTGGTCGCATA 3' R - 5' CGAATGCGCAGCACCAG 3'	(Poirel *et al.,* 2011)	390
2	*bla*NDM – 1	F - 5' GGTTTGGCGATCTGGTTTTC 3' R - 5' CGGAATGGCTCATCACGATC 3'	(Poirel *et al.,* 2011)	621
3	*bla*CTX-M-1	F - 5' AAAAATCACTGCGCCAGTTC 3' R - 5' AGCTTATTCATCGCCACGTT 3'	(Woodford *et al.,* 2006)	415
4	*bla*TEM – 1	F - 5' CCGAAGAACGTTTTCCAATG 3' R - 5' GTCCTCCGATCGTTGTCAGAA 3'	(Jiménez-Castellanos *et al.,* 2018)	249

Extraction of bacterial genomic DNA (Using PrimeWay genomic DNA extraction kit)

Klebsiella spp. were cultured on LB agar plates at 37°C overnight. A few colonies from the overnight culture were transferred to a microcentrifuge tube containing 100 µL of sterile deionized water. DNA was extracted using the PrimeWay Genomic DNA Extraction Kit (1st BASE,Malaysia), following the manufacturer's protocol.

Quantification of genomic DNA

The quality of the extracted DNA was assessed by gel electrophoresis on a 1% (w/v) agarose gel, where the integrity of the DNA was evaluated based on the size and clarity of the DNA bands. For quantification, the total DNA concentration was measured using an IMPLEN NanoPhotometer™. The purity of the DNA was determined by the (260/280) absorbance ratio, which ranged from 1.8 to 2.0 for all samples.

RESULTS

Antibiotic susceptibility and resistance profiles of Klebsiella spp. clinical isolates

A total of 85 *Klebsiella* spp. clinical isolates were obtained from various sources as shown in Figure 1 from January 2022 to March 2023. Although the source samples were diverse, most samples were obtained from urine (45%), followed by sputum (20%) and blood (18%). Less than 6% of the samples were obtained from wound, rectal swab, tracheal aspirate, pus, bronchoalveolar lavage and tissue. The results of the disk diffusion assay were interpreted by measuring the diameter of the zone of inhibition for each antibiotic and classifying them as susceptible, intermediate, or resistant according to CLSI M100 susceptibility breakpoints (CLSI,2020). Based on Figure 2, the tested bacterial strain showed resistance towards ampicillin as it did not exhibit a clear zone of inhibition. The 85 clinical isolate's antibiotic susceptibility profiles were shown in Table 2.

All 85 strains of *Klebsiella* spp. tested demonstrated resistance towards ampicillin and ciprofloxacin (Table 2, Figure 3). Only 17 (20%) were resistant to chloramphenicol. On average, 50 strains (59%) were resistant towards cephalosporins, indicative of ESBL-producing strains. Next, 51 strains (60%) were non-susceptible towards imipenem and 68 strains (80%) were non-susceptible towards meropenem, indicative of CRE producing strains. Apart from beta-lactams, 44 strains (52%) were resistant to kanamycin. The isolates from urine had a high level of resistance to cephalosporins, followed by sputum and blood. On contrary, isolates from blood, urine and sputum displayed less resistance to chloramphenicol.

Therefore, the results indicated that most of the strains exhibited resistance towards cephalosporins and carbapenems. We suspected that these strains harboured ESBL and CRE genes. To confirm this, we selected several strains for amplification of the ESBL genes such as the TEM-1 and CTXM-1 to detect the presence of the genes followed by NDM-1 and VIM-1 to detect carbapenem genes.

Detection of ESBLs and carbapenemases genes through PCR

Among the six selected strains for further identification of the presence of *bla*TEM, *bla*CTX-M, *bla*VIM, and *bla*NDM genes, strains 65, 64, and 15 exhibited resistance to all classes of antibiotics tested. Strain 15 was resistant to all antibiotics tested except chloramphenicol, while strain 18 was resistant to cephalosporins and other classes of antibiotics except imipenem and meropenem, which were carbapenems. Additionally, strain 23 demonstrated susceptibility to cefuroxime and cefotaxime but resistance to ceftazidime, along with susceptibility to carbapenems, chloramphenicol, and norfloxacin.

Results revealed that all the tested strains, showed the presence of TEM-1 gene (Figure 4(A)). Next, 5 strains showed the presence of CTX-M-1 (Figure 4(B)) and those strains were resistant towards cefotaxime, ceftriaxone a third-generation cephalosporin and ceftazidime, a fourth-generation cephalosporin. Bands were present in strains that were intermediate to cephalosporins whereas no visible bands were observed in strain number 23 which were susceptible towards cefuroxime, cefotaxime, and intermediate to ceftazidime.

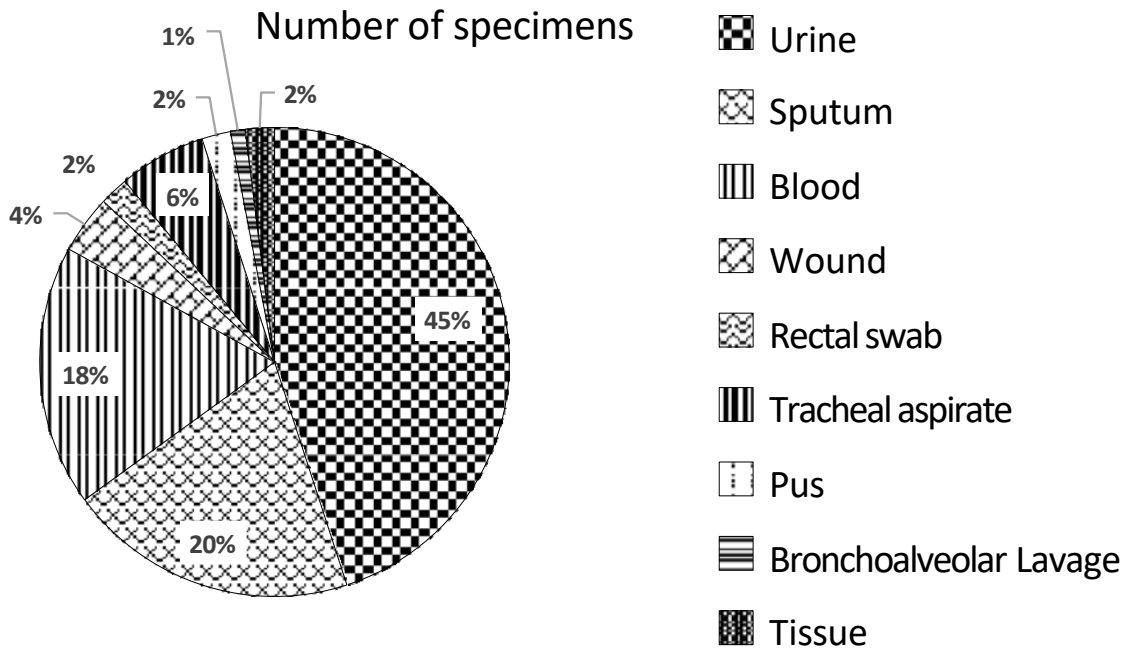

Figure 1. Percentage of *Klebsiella* spp. isolated from various clinical specimens.

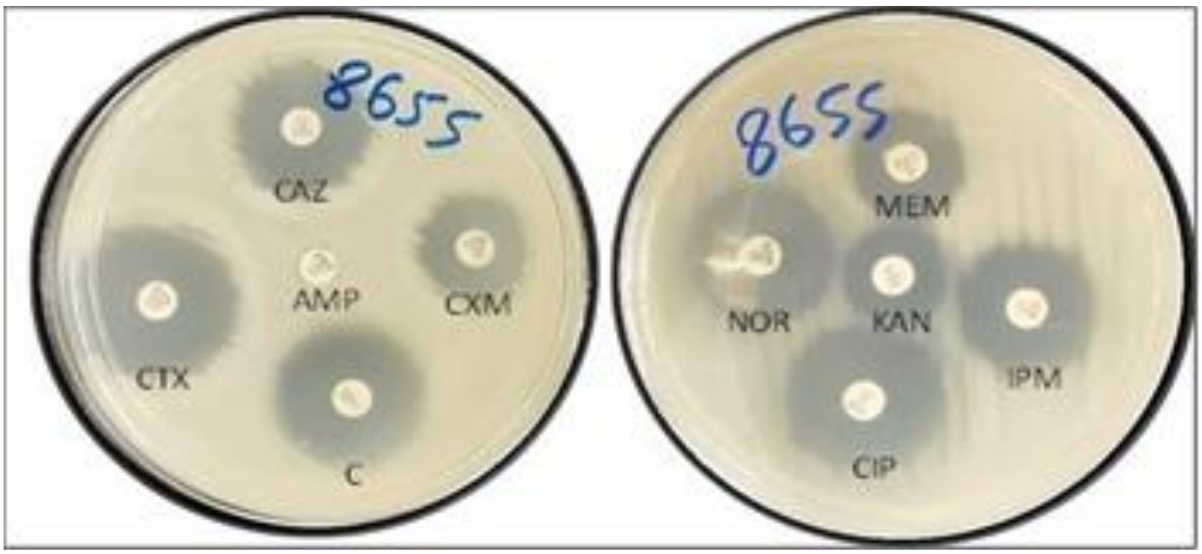

Figure 2. Representative figure shows the disk diffusion assay results for clinical isolates exhibiting resistance to ampicillin. The figure corresponds to Isolate No.41 as detailed in Table 2. The figure is a snapshot of a broader study, detailed methodology and conditions are provided in accompanying methods section. Abbreviations: CXM, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; IPM, imipenem; MEM, meropenem; AMP, ampicillin; K, kanamycin; C, chloramphenicol; CIP, ciprofloxacin; NOR, norfloxacin

Table 2. Results of the disk diffusion assay showing antibiotic susceptibility profile of 85 clinical isolates. The table includes the diameter of inhibitory zones measured in millimetres (mm) for each antibiotic tested, interpreted as susceptible, intermediate, or resistant according to CLSI guidelines.

No	Sources/Specimen	CXM	CTX	CAZ	IPM	MEM	AMP	KAN	C	CIP	NOR
1	Urine	6	6	6	22	15	6	6	16	6	6
2	Urine	6	6	6	23	18	6	6	6	7	6
3	Urine	6	6	10	20	17	6	16	19	6	15
4	Sputum	6	6	6	21	17	6	15	6	9	16
5	Sputum	6	6	12	19	18	6	12	17	17	18
6	Sputum	6	9	9	19	18	6	18	17	12	19
7	Urine	6	8	11	20	16	6	12	20	16	10
8	Urine	6	6	6	23	16	6	6	23	6	6
9	Blood	6	9	13	22	27	6	17	20	20	17
10	Urine	6	6	11	17	23	6	6	6	6	6
11	Tracheal Aspirate	6	6	9	21	23	6	6	19	6	6
12	Tracheal Aspirate	6	6	9	22	23	6	6	20	6	6
13	Urine	6	6	6	22	23	6	6	6	6	6
14	Urine	6	6	9	21.5	23	6	6	6	13	12
15	Tracheal Aspirate	6	6	10	22	18	6	16	6	18	14
16	Sputum	14	14	10	25	26	6	16	25	20	21
17	Urine	6	10	13	21	25	6	6	6	6	6
18	Sputum	6	6	6	23	28	6	6	6	6	6
19	Blood	6	6	6	21	20	6	6	16	6	6
20	Sputum	6	6	6	17	20	6	6	17	6	6
21	Urine	6	9	17	24	24	6	16	20	9	17
22	Urine	14	26	21	22	25	6	6	6	20	19
23	Sputum	20	26	24	24	23	8	17	24	20	22
24	Urine	25	34	32	27	32	6	24	21	20	22
25	Urine	6	6	11	31	32	6	8	23	6	6
26	Sputum	18	24	20	20	21	6	15	24	20	20
27	Sputum	6	7	11	17	18	6	6	23	6	11
28	Urine	16	17	19	20	20	6	18	16	12	13
29	Urine	15	26	19	20	20	6	14	22	17	17
30	Urine	6	6	10	16	18	6	6	23	6	11
31	Sputum	16	24	20	22	20	6	14	22	20	29
32	Urine	6	6	8	21	19	6	6	21	6	6
33	Urine	6	8	13	22	17	6	18	18	20	23
34	Tracheal Aspirate	6	6	13	22	19	6	19	24	12	17
35	Urine	15	26	19	21	20	6	16	16	11	16
36	Urine	6	19	18	25	13	6	12	20	16	18
37	Blood	18	24	19	22	18	6	15	23	13	15
38	Blood	17	24	19	21	18	6	14	18	18	19
39	Sputum	13	24	18	23	17	6	9	21	19	20
40	Urine	18	25	21	25	19	6	15	21	18	20
41	Urine	19	24	20	23	17	6	14	23	20	20
42	Pus	19	24	20	22	17	6	12	21	19	20
43	Tissue	18	22	18	23	23	6	14	20	20	20
44	Urine	14	22	15	23	17	6	13	16	17	16
45	Urine	6	6	8	20	16	6	12	22	20	17
46	Blood	19	20	15	12	17	6	16	23	20	14
47	Urine	19	24	21	24	24	6	17	24	20	20
48	Blood	12	15	18	20	22	6	16	12	18	15
49	Wound	6	6	11	17	18	6	18	18	16	22
50	Urine	6	6	8	21	21	6	14	19	15	15
51	Urine	6	6	6	23	23	6	6	22	16	15
52	Urine	6	8	11	24	21	6	12	13	21	19
53	Wound	18	23	23	22	20	6	12	22	10	17
54	Urine	6	6	6	21	17	6	6	16	6	6
55	Urine	6	10	15	21	21	6	19	23	20	24
56	Urine	19	25	21	24	20	7	15	23	20	21
57	Blood	13	21	16	20	21	6	6	6	15	15
58	Rectal Swab	6	6	6	21	11	7	6	20	6	6
59	Rectal Swab	6	6	6	21	9	6	6	20	6	6
60	Sputum	16	22	19	22	16	6	15	21	20	19
61	Sputum	15	20	18	20	16	6	6	6	13	12
62	Urine	19	24	21	23	20	8	18	22	20	23
63	Sputum	17	23	19	20	15	7	15	22	18	18

64	Bal	6	18	15	16	13	6	13	6	15	14
65	Blood	6	17	14	18	15	6	7	6	13	13
66	Blood	20	25	20	23	20	6	16	24	20	20
67	Urine	6	10	16	24	19	6	6	6	6	6
68	Blood	20	28	22	23	21	6	18	23	20	22
69	Blood	13	24	20	23	20	6	9	15	15	17
70	Blood	6	10	16	24	19	6	8	6	6	6
71	Blood	20	28	22	23	21	6	18	23	20	22
72	Urine	13	24	20	23	20	6	9	15	15	17
73	Blood	6	10	16	24	19	6	8	6	6	6
74	Urine	19	25	22	25	18	6	19	22	20	23
75	Sputum	19	26	22	23	19	6	17	23	21	23
76	Sputum	20	25	23	23	19	6	17	25	20	22
77	Blood	17	24	21	21	17	6	15	22	21	20
78	Pus	19	24	22	23	17	6	16	24	21	22
79	Urine	19	27	21	23	17	6	15	24	21	18
80	Urine	18	25	22	22	18	6	15	23	20	22
81	Urine	18	25	22	21	18	6	16	23	21	22
82	Wound	17	26	19	23	16	6	14	22	17	16
83	Sputum	20	24	21	21	16	6	16	22	21	21
84	Tissue	21	24	22	15	18	6	15	12	21	24
85	Tracheal Aspirate	20	25	23	23	19	6	17	25	21	22

Abbreviations: CXM, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; IPM, imipenem; MEM, meropenem; AMP, ampicillin; K, kanamycin; C, chloramphenicol; CIP, ciprofloxacin; NOR, norfloxacin. The diameters of the zone of inhibition were determined as per the M100 guidelines and interpreted as susceptible (green), intermediate (yellow) and resistant (red) (CLSI,2020)

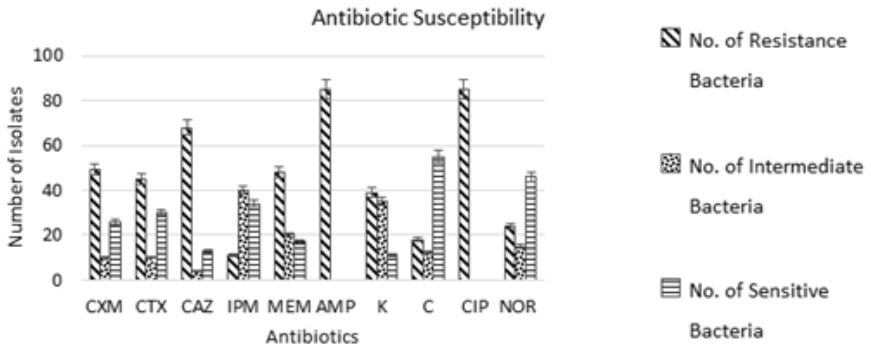

Figure 3. Number of bacterial strains based on antibiotic susceptibility for each antibiotic. Abbreviations: CXM, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; IPM, imipenem; MEM, meropenem; AMP, ampicillin; K, kanamycin; C, chloramphenicol; CIP, ciprofloxacin; NOR, norfloxacin

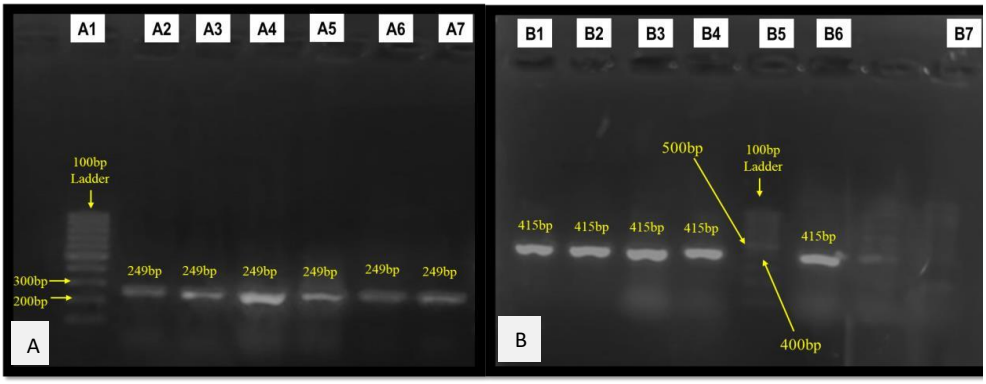

Figure 4. Gel electrophoresis of *blaTEM-1*(A) and *blaCTX-M-1*(B) PCR products. Lane A1&B5: 100bp DNA ladder (Generuler™, Thermo Fisher Scientific); Lanes A2-A7: *blaTEM-1* PCR products (249bp), corresponding to strains 15, 18, 27, 64, 65, and 23, respectively; Lanes B1-B4 & B6: *blaCTX-M-1* PCR products (415bp), corresponding to strains 15, 18, 27, 64, and 65; Lane B7*: blaCTX-M-1* PCR products with no band, corresponding to strain 23.

Subsequently, the same strains as used for testing TEM-1 and CTXM-1 were selected to test for the NDM-1 and VIM-1 genes. Among these strains, some were susceptible to imipenem and meropenem, while others were resistant. Strains 15, 27, 64, and 65 showed the presence of NDM genes, as depicted in (Figure 5(A)) resulting in resistance towards meropenem, imipenem or both antibiotics. Additionally, the study revealed the presence of the VIM gene in these same strains (15, 27, 64, and 65), as shown in Figure 5(B), correlating with their resistance to carbapenems. Notably, strain numbers 18 and 23, which were susceptible to imipenem and meropenem, showed no visible bands when tested for NDM and VIM genes.

DISCUSSION

Klebsiella-associated infections have emerged as a significant challenge in healthcare settings, driven by the increasing prevalence of MDR strains. These resistant strains limit the effectiveness of commonly used antibiotics, posing a serious threat to patient outcomes, particularly in vulnerable populations (Serwecińska, 2020). The high prevalence of ESBL-producing *K. pneumoniae* can likely be attributed to its nosocomial nature and the presence of diverse replicons carried by MDR strains (Al-Marzooq, 2015). The mechanisms of drug resistance have been linked to various chromosomal and plasmid-encoded beta lactamase genes (Carlet,2012). In HSAAS, a significant proportion of *Klebsiella* strains were isolated from various clinical samples and regardless of the isolation source, all strains demonstrated complete resistance to ampicillin and ciprofloxacin. Although the presence of ESBL genes are known to causes resistance to beta lactam particularly the cephalosporins, resistance to cephalosporins were also detected. This could be due to mutations in the gyrA genes (Hooper, 2001; Seiffert,2013). These highlight the urgent need for robust antibiotic stewardship programs to limit the misuse of antibiotics and prioritize their appropriate use (Barlam, 2021). Routine molecular screening, such as multiplex PCR to detect the presence of these beta lactamase genes, should already be implemented in healthcare settings to track resistance patterns and guide empirical therapy. In fact, detection of antimicrobial resistance gene and possible markers associated with drug resistance using MALDI-TOF Mass Spectrometry should be employed in hospitals for rapid detection of antimicrobial resistance in bacterial pathogens (Florio *et al.*, 2020). Additionally, enhanced infection control measures, such as stringent hygiene protocols and isolation of infected patients, are important to control the spread of these resistant strains (Yang *et al.*, 2023).

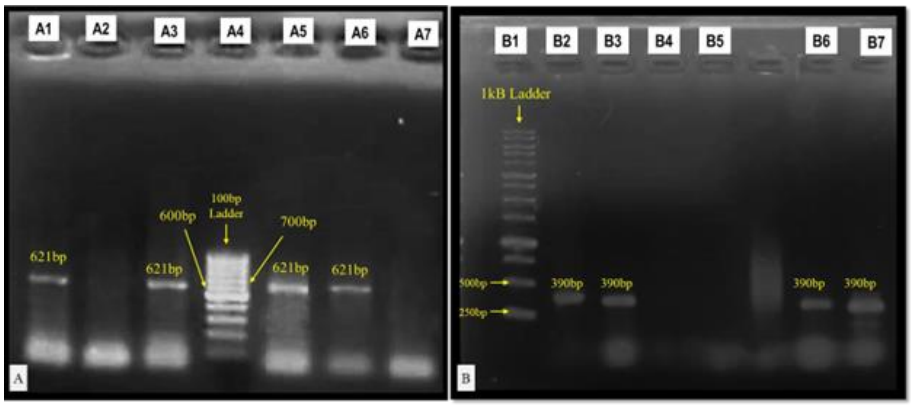

Figure 5. Gel electrophoresis of *blaNDM*(A) and *blaVIM*(B) PCR products. Lane A4: 100bp DNA ladder (Generuler™, Thermo Fisher Scientific); Lane B1: 1kb DNA ladder (Exact Mark, 1st BASE Pte Ltd); Lanes A1, A3, A5, and A6: *blaNDM* PCR products (621bp), corresponding to strains 15, 27, 64, and 65, respectively; Lanes A2 & A7: *blaNDM* PCR products with no band, corresponding to strains 18 and 23; Lanes B2, B3, B6, and B7: *blaVIM* PCR products (390bp), corresponding to strains 15, 27, 64, and 65, respectively; Lanes B4 & B5: *blaVIM* PCR products with no band, corresponding to strains 18 and 23.

The detection of *bla*-TEM and *bla* CTX-M genes in *Klebsiella* clinical isolates highlights their significant role in mediating β-lactam resistance, particularly against extended-spectrum cephalon-sporins like cefotaxime and ceftriaxone. CTX-M enzymes are categorized into five primary groups based on sequence homology: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25, as well as CTX-M-151 as the new cluster (Mendoca *et al.,* 2022). Among these, the most frequently reported variants worldwide include CTX-M-15 (CTX-M-1 group), which is dominant in nosocomial settings, CTX-M-3 (CTX-M-1 group), and CTX-M-14 (CTX-M-9 group) (Bonnet,2004). In recent years, CTX-M-27 (CTX-M-9 group) has also been increasingly reported. These enzymes exhibit evolving hydrolytic activity, particularly against third-generation cephalon-sporins such as cefotaxime and ceftriaxone, with CTX-M-15 and CTX-M-27 variants demo-nstrating enhanced activity against ceftazidime. Furthermore, infections caused by CTX-M-15-producing *K. pneumoniae* have shown a marked increase in nosocomial settings over recent years (Lim *et al*., 2009; Lee *et al*., 2011). In Malaysia, as in other Asian countries, CTX-M-15 is the major ESBL variant reported in *K. pneumoniae* isolates, often contributing to multidrug resistance and posing significant challenges for clinical management (Lim *et al*., 2009; Lee *et al*., 2011; Mohd Helmi *et al*., 2016). The widespread occurrence of *bla*CTX-M strains complicates infection treatment in hospital settings, increasing the likelihood of therapeutic failure. Besides that, TEM-1 further complicates infection management by conferring resistance to penicillin and ampicillin, with the TEM-1 beta-lactamase enzyme hydrolyzing the beta-lactam ring of these antibiotics, rendering them ineffective. This gene is commonly found in clinical isolates of *K. pneumoniae* and other Gram-negative bacteria, making it a significant contributor to hospital-acquired infections (Caneiras *et al*., 2019).

NDM-1 and VIM-1 are metallo-beta-lactamases that confer resistance to carbapenems, presenting a significant challenge in treating infections caused by *K. pneumoniae* and other Gram-negative bacteria. NDM-1 is of particular concern due to its ability to hydrolyze carbapenems and other beta-lactams, drastically limiting treatment options for infections that are difficult to treat with traditional antibiotics (Lau *et al*., 2024). Its prevalence is rapidly increasing, particularly in hospital-associated infections, with significant occurrences in regions such as South Asia, Europe, and the Middle East (Huttner *et al*., 2013). In Malaysia, there have been only a few reports on the identification and characterization of NDM-1 carbapenamase. In one of the study, NDM-1 carbapenemase was detected in three *K. pneumoniae* isolates, while no carbapenemase genes were found in any of the *E. coli* isolates. The three NDM-1-positive strains were also positive for at least one other β-lactamase gene (CTX-M, SHV, or TEM), which classifies them as MDR bacteria. The three NDM-1-positive isolates exhibited resistance to nearly all the tested antibiotics, except for carbapenems, but were susceptible to polymyxin B (Ahmad *et al*., 2013). Therefore, this finding is consistent with many studies worldwide, which show that NDM-1 positive strains are usually resistant to all antibiotics except polymyxins (Molton *et al*., 2013). Moreover, in a study monitoring resistance trend among *Enterobacteriaceae* isolates in the Asia-Pacific region, NDM-1 was identified as the most common carbapenemase (Grover *et al*., 2017). Similarly, VIM-1, which also provides resistance to carbapenems, complicates treatment regimens in healthcare settings (Ngoi *et al*., 2021). In a study conducted on CRE isolated from infected patients at Dr. Mohammad Hoesin Hospital, Palembang, Indonesia, it was found that 85.7% of the isolates lacking the *bla*VIM gene still exhibited resistance to ertapenem or meropenem. This suggests that resistance in these isolates may arise through alternative mechanisms, including the expression of other carbapenemase genes or non-enzymatic mechanisms such as porin alterations, reduced drug penetration to penicillin-binding proteins (PBPs), or increased efflux pump activity (Amalia *et al*., 2019).

The findings of this study highlight the alarming prevalence of ESBL- and CRE-producing *Klebsiella* spp. isolates, posing a significant public health concern in Malaysia. Reports indicate that ESBL-producing *K. pneumoniae* is prevalent in 30-50% of Malaysian hospitals, with carbapenem resistance rates steadily increasing (Kong *et al*., 2022). These statistics emphasize the urgent need for effective interventions to combat the spread of these

resistant strains. Key strategies include optimizing antibiotic selection and implementing robust infection control measures. The integration of Clinical Decision Support Systems (CDSS) and Antibiotic Stewardship Programs (ASP) can guide the rational use of antibiotics, minimize selective pressure, and prevent the emergence of resistance (Ng *et al*., 2022 ; Ya *et al*.,2023).

To address the growing threat of ESBL and CRE-producing *Klebsiella* spp., a variety of strategies are necessary. Alternative treatments, including newer β-lactam/β-lactamase inhibitor combinations, such as ceftazidime-avibactam, or non-β-lactam antibiotics, should be considered (Lee *et al*., 2003; Yahav *et al*., 2020). Additionally, novel therapeutic approaches, such as antimicrobial peptides or combination therapies, may offer promising solutions. The findings of this study provide valuable insights into the resistance mechanisms of *Klebsiella* spp., contributing to informed antibiotic prescription practices and reducing the risk of therapeutic failures. Moreover, this study highlights the need for advanced diagnostic tools to rapidly identify resistance genes, enabling more targeted treatment strategies. These results can also inform national surveillance programs and support policy development to combat antimicrobial resistance on a broader scale.

There were several limitations in this study. First, this analysis targeted specific alleles of certain resistance genes (*bla*CTX-M, *bla*TEM-1, *bla*NDM-1 and *bla*VIM- 1) using PCR primers, potentially overlooking other variants of resistance. In addition, advanced techniques such as whole-genome sequencing were not used, limiting our ability to comprehensively analyze resistance mechanisms. Despite these limitations, our findings highlight the decreased susceptibility of *Klebsiella* isolates to most used antibiotics, focusing the importance of selecting appropriate antibiotics and implementing effective infection control measures to manage infections caused by these resistant strains.

CONCLUSION

The rise in MDR *Klebsiella* spp. is continuing to pose a serious threat to healthcare. Therefore, periodic monitoring through antibiotic surveillance is necessary for improvement in patient care. In this study, all *Klebsiella* spp. isolates from hospital patients exhibited high resistance against different classes of antibiotics. Isolates harbouring the *TEM* and *CTX-M* genes were found responsible for ESBL production while the presence of *VIM* and *NDM* genes possibly indicates that the strains are CRE- producers. Moving forward, a comprehensive understanding of antimicrobial resistance trends and surveillance among other hospitals on the genetic diversity of ESBLs and CRE is essential for the development of effective treatment strategies.

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CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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