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The Molecular Characteristics of Extended-Spectrum β-Lactamases (ESBL), Carbapenem-Resistant Enterobacterales (CRE) and Susceptible Isolates of *Klebsiella pneumoniae* and *Escherichia coli* in Hospital Pakar Universiti Sains Malaysia (HPUSM), Kelantan, Malaysia

(Pencirian Molekul Spektrum Lanjutan β-Lactamases (ESBL), Carbapenem-Rintang Enterobacterales (CRE) serta Pencilan Kerentanan *Klebsiella pneumoniae* dan *Escherichia coli* di Hospital Pakar Universiti Sains Malaysia (HPUSM), Kelantan, Malaysia)

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ABSTRACT

Multidrug-resistance *Klebsiella pneumoniae* (MDR-KP) has become a major challenge to clinicians as it caused significant morbidity and mortality among hospitalized patients. This study aims to determine the antibiotic susceptibility profiles of 17 *K. pneumoniae* strains isolated to different antimicrobial agents and to characterize the genes encoding extended-spectrum β-lactamase (ESBL), AmpC and (Carbapenem Resistance Enterobacteriacae (CRE) phenotypes by using VITEK AST (Antimicrobial Susceptibility Test), phenotypic disk confirmatory test (PDCT) and polymerase chain reaction (PCR). Out of 17 *K. pneumoniae* isolates tested, seven (41.2%) were confirmed to be ESBL producers, carrying bla_{TEM} (1; 14.3%), bla_{SHV} (1; 14.3%), bla_{CTXM-1} (4; 57.14%), $bla_{CTXM-14}$ (2; 28.6%) and co-existence of both bla_{TEM} and bla_{CTXM-1} (1; 14.3%) genes, while four *K. pneumoniae* (23.5%) isolates were CRE strains, carrying co-existence of bla_{TEM} and bla_{CTXM-1} genes, as well as bla_{NDM-1} (4; 100%). bla_{TEM} and bla_{CTXM} genes were the most predominant genes detected in both *K. pneumoniae* ESBL and CRE isolates and bla_{NDM-1} genes was detected in *K. pneumoniae* CRE isolates which were in line with other findings worldwide. Understanding this link highlights the need for strategic antibiotic usage in healthcare settings by providing a deeper understanding of antibiotic resistance trends in multidrug resistant (MDR) organisms.

Keywords: Antibiotic resistant; CRE; ESBL; *Klebsiella pneumoniae*

ABSTRAK

Klebsiella pneumoniae rintang pelbagai ubat *(MDR-KP)* telah menjadi cabaran utama bagi pakar klinikal kerana ia menyebabkan morbiditi dan kematian yang signifikan dalam kalangan pesakit yang telah dirawat di hospital. Penyelidikan ini bertujuan untuk menentukan profil kerentanan antibiotik bagi 17 strain *K. pneumoniae* yang dipencilkan kepada agen antimikrob yang berbeza dan untuk mencirikan gen beta-laktamase spektrum lanjutan (ESBL), AmpC dan *Enterobcacteriacae* perintang karbapenem (CRE) menggunakan VITEK AST (Ujian Kecenderungan Antimikrob), ujian pengesahan cakera fenotipik (PDCT) dan reaksi rantaian polimerase (PCR). Daripada 17 pencilan *K. pneumoniae* yang diuji, tujuh pencilan (41.2%) telah disahkan sebagai pengeluar ESBL, membawa gen bla_{TEM} (1; 14.3%), bla_{SHV} (1; 14.3%), bla_{CTXM-1} (4; 57.14%), $bla_{CTXM-14}$ (2; 28.6%) serta kewujudan bersama bla_{TEM} dan bla_{CTXM-1} (1; 14,5%), manakala empat pencilan CRE (23.5%) membawa kewujudan bersama gen $bla_{TEM,}$ bla_{CTXM-1} serta bla_{NDM-1} (4, 100%). Gen bla_{TEM} dan bla_{CTXM} adalah gen yang paling dominan yang dikesan dalam kedua-dua pencilan *K. pneumoniae* ESBL dan CRE serta bla_{NDM-1} telah ditemui dalam *K. pneumoniae* CRE dan keputusan ini adalah sejajar dengan penemuan lain di seluruh dunia. Memahami hubungan antara kedua-dua ini menyerlahkan kepentingan penggunaan antibiotik secara strategik dalam penjagaan kesihatan dengan menyediakan pemahaman yang lebih mendalam mengenai kerintangan antibiotik dalam organisma yang rintang pelbagai ubat (MDR).

Kata kunci: CRE; ESBL; *Klebsiella pneumoniae*; rintang antibiotik

INTRODUCTION

Klebsiella pneumoniae is one of the most common Gram-negative bacteria encountered by physicians worldwide, accounting for 0.5-5.0% of all pneumonia cases (Ashurst & Dawson 2022). *K. pneumoniae* is universally considered as nosocomial pathogen, a leading cause of hospital-acquired pneumonia, bacteraemia, septicaemia (blood), meningitis (brain), endocarditis (heart), cellulitis (skin) and urinary tract infections (UTIs). Resultant infections are associated with prolonged hospital stay and high mortality rates up to 50%-100% especially in patients with alcoholism and septicaemia (Ashurst & Dawson 2022; Esposito et al. 2018; Walter et al. 2018). In Malaysia, a study conducted at Hospital Canselor Tuanku Muhriz Universiti Kebangsaan Malaysia reported a relatively low mortality rate of 12.3% in *K. pneumoniae* bacteraemia as compared to the other regions like United States and Portugal (Ang et al. 2019; Caneiras et al. 2019; Magill et al. 2014). Nevertheless, another previously published study reported that the carrier rates for *K. pneumoniae* was as high as 77% and commonly related to the number of antibiotics administered (Ashurst & Dawson 2022).

According to a recent review article on nano-antibiotics (Li et al. 2023), multidrug-resistance (MDR) can be defined as insusceptibility (resistant) to at least one agent from three or more antibiotic classes. *K. pneumoniae* was previously identified as one of the most common organisms capable of producing extended-spectrum β-lactamase (ESBL) enzyme. ESBLs are define as β-lactamases that confer resistance to first, second and third generation cephalosporins, aztreonam (but not cephamycin or carbapenem) by causing hydrolysis and inhibited by clavulanic acid which is a β-lactamase inhibitor (Paterson & Bonomo 2005). ESBLs have spread widely and in some countries, a significant proportion of *Escherichia coli* strains now harbour ESBL enzyme, which reinforces the widespread nature of this resistance mechanism across different bacterial species too (Candan & Aksöz 2015; Fils et al. 2021).

Compounding to this issue, multidrug resistance *Klebsiella pneumoniae* has become a major challenge to clinicians and researchers as it becomes resistant to many β-lactam antibiotics including carbapenem, which was used as a 'last-line' defense antibiotic (Leavitt et al. 2009; Nordmann, Dortet & & Poirel 2012; Pitout, Nordmann & Poirel 2015; Wu et al. 2011). Carbapenem resistant Enterobacterales (CRE) were defined as Enterobacterales that test resistant to at least one of the carbapenem antibiotics (ertapenem (MIC: ≥2), meropenem (MIC: ≥4), doripenem (MIC: ≥4), or imipenem (MIC: ≥4)) or that produce an enzyme called carbapenemase (which can render them resistant to carbapenem antibiotics) identified either by phenotypic or molecular tests (Center for Disease Control and Prevention 2019; Clinical & Laboratory Standard Institute 2023).

Although similar studies examining ESBL and CRE mechanisms in *K. pneumoniae* and *E. coli* have been conducted in various settings (Karaman et al. 2024; Pishtiwan & Khadija 2019), it is well-recognized that antibiotic resistance patterns and the distribution of resistance genes vary significantly by geographic region and also between hospitals even when it is located within the same country. Differences in antibiotic stewardship practices, infection control measures and patient demographic might contribute to these variations. Therefore, a comprehensive surveillance for targeted MDR organism must be implemented to reduce the rate of transmission and further understanding regional resistance trends. In this context, present study aims to assess the antibiotic susceptibility profiles and characterize the genes encoding the ESBL and CRE phenotypes among clinical isolates *K. pneumoniae* and *E. coli* from HPUSM, Kelantan. Our finding aims to enhance the broader understanding of antimicrobial resistance trends in Malaysia and to emphasize the critical needs of implementing hospital-specific resistance surveillance programs.

MATERIALS AND METHODS

ETHICS

This study protocol was approved by institutional ethical committee (ID number: USM/JEPeM/KK/23040335) prior to commencement. No patient's consent was needed since this study only involved archived specimens. The privacy of the patients was ensured by labelling the samples with laboratory codes rather than the name.

BACTERIAL ISOLATES AND IDENTIFICATION

The study was performed between January 2023 and October 2023 in Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia. A total of 24 *Klebsiella pneumoniae* and *Escherichia coli* isolates were obtained from archived biobank of INFORMM, Universiti Science Malaysia. Isolates were recovered from variety of specimen types and susceptibility (Table 1). The isolates were then cultured and further confirmed using Triple Sugar Iron (TSI) biochemical test. *Escherichia coli* and *Klebsiella pneumoniae* ATCC 1705 were used as a quality control in this study.

ANTIMICROBIAL SUSCEPTIBILITY TESTING (AST)

Rapid antimicrobial susceptibility testing (AST) was performed using VITEK 2 (bioMerieux, France) according to manufacturer's recommendation. Bacteria were grown on MacConkey agar and incubated overnight at 37 ºC. 0.50-0.63 McFarland suspension for Gram-negative bacilli was prepared in 0.45% of saline solution and the reading was confirmed using nephelometer. Minimum inhibitory concentration (MIC) value of the samples was interpretated in accordance with Clinical and Laboratory Standards Institute (CLSI) M100 guideline (33rd ed. 2023). Findings

TABLE 1. Sources of the *Klebsiella pneumoniae* and *Escherichia coli* isolates

No	Organism	Lab No	Type of Specimen	Susceptibility
1.	*Klebsiella pneumoniae* ESBL	U39167	Urine	Resistant
2.		P39441	Pus Aspirate	Resistant
3.		P39598	Pus Swab	Resistant
4.		B40069	Blood	Resistant
5.		B40729	Blood	Resistant
6.		B40889	Blood	Resistant
7.	*Klebsiella pneumoniae* CRE	SP39656	Sputum	Resistant
8.		P40167	Pus Aspirate	Resistant
9.		B36336	Blood	Resistant
10.		U39122	Urine	Resistant
11.		B38211	Blood	Resistant
12.	*Klebsiella pneumoniae* Susceptible*	23020656	Pus	Susceptible
13.		23023184	Sputum	Susceptible
14.		23020666	Pus	Susceptible
15.		23020662	Pus	Susceptible
16.		23023064	Blood	Susceptible
17.		23020772	Pus	Susceptible
18.	*Escherichia coli*	B41398	Blood	Susceptible
19.		U49154	Urine	Susceptible
20.		23026285	Pus	Susceptible
21.		23024821	Blood	Susceptible
22.		23026478	Blood	Susceptible
23.		23025438	Blood	Susceptible
24.	*Klebsiella pneumoniae* (Control)	ATCC 1705	-	Resistant

ESBL: Extended-Spectrum-Beta-Lactamases, CRE: Carbapenem-resistant Enterobacterales

*"Susceptible' refers to *Klebsiella pneumoniae* isolates that were neither ESBL producers nor Carbapenem-resistant (CRE) strains

were classified as susceptible, susceptible-dose dependent (sdd)/intermediate or resistant based on MIC breakpoints and zone diameter interpretive criteria established by CLSI. An isolate was considered susceptible if it was inhibited by the usually achievable concentrations of antimicrobial agent when the recommended dosage was introduced, indicating likely clinical efficacy. Furthermore, according to CLSI, the susceptible-dose dependent (SDD) can be referred to isolates for which clinical efficacy depended on achieving higher dosing regimens that provided greater drug exposure than the standard susceptible breakpoint. On the other hand, intermediate category defined by a breakpoint that includes isolates with MICs or zone diameters approached attainable blood or tissue levels and response rates were potentially lower compared to susceptible isolates. Lastly, resistant can be defined as isolates that was not inhibited by usually achievable concentrations of the antimicrobial agent under normal dosage regimens (Clinical & Laboratory Standard Institute 2023).

CARBAPENEM-RESISTANT ENTEROBACTERALES (CRE) AND EXTENDED-SPECTRUM-BETA-LACTAMASES (ESBL) SCREENING AND CONFIRMATORY TEST

Klebsiella pneumoniae isolates were examined for phenotypic expression of ESBLs, AmpCs, and cabapenemases. Detection of carbapenem resistant was confirmed using minimum inhibitory concentration (MIC) of carbapenem antibiotics (ertapenem, meropenem and imipenem) either via VITEK AST or E-test according to manufacturer's instructions. Isolates were classified as carbapenem-resistant if the MIC was ≥2 µg/mL for ertapenem, or ≥4 µg/mL for meropenem and imipenem. Isolates that were resistant to at least one of the carbapenem antibiotics will be considered as CRE (Clinical & Laboratory Standard Institute 2023).

Phenotypic screening method using ceftazidime (30 µg) and cefotaxime (30 µg) antibiotics were used to determine the inhibition zone for all samples to identify possible ESBL-producing strains. Inhibition

zone ≤ 22 mm and ≤ 27 mm for both ceftazidime and cefotaxime, respectively, will be considered as a possible ESBL-producing strains.

Phenotypic disk confirmatory test (PDCT) was then used to further confirm the ESBL-producing isolates. Ceftazidime (30 mcg), ceftazidime-clavulanic acid (30/10 mcg), cefotaxime (30 mcg), cefotaxime-clavulanic acid (30/10 mcg) and cefoxitin (30 mcg) discs were used as the indicator to confirm the ESBL strains. A potentiation of the inhibition zone (≥ 5mm zone diameter) of any antibiotic tested in combination with clavulanic acid versus single antibiotic (either cefotaxime or ceftazidime) will be considered as positive results and recorded as ESBL-producing strains.

POLYMERASE CHAIN REACTION AMPLIFICATION

DNA templates of the isolates were prepared using QIAamp DNA Blood Mini Kit (QIAGEN Inc, Valencia, CA, USA) according to the manufacturer's instructions. Concentration of DNA template was then determined using NanoDrop ® ND-1000 spectrophotometer (Wilmington, DE, USA). Primers for the targeted genes were used in a combination of singleplex and multiplex PCR for molecular characterization as listed in Table 2.

PCR was performed in reaction mixes with a volume of 25 µL that contained 0.5 µM of each primer (as indicated in Table 2), 1.0 µL of DNA templates, 1x Green Go Taq Polymerase, 1.5 mM $MgCl_2$, 0.2 µM dNTP and 0.04 U/µL of Taq polymerase in the reaction buffer provided by the manufacturer (Promega, USA). Amplification condition was set up under the following conditions: initial denaturation at 10 min for 94 ºC, followed by 30 - 45 cycles of denaturation for 1 min at 94 ºC, primers annealing at specific temperature (Table 2), extension for 1 min at 72 ºC with a final extension period of 10 min at 72 ºC in a DNA thermal cycler (Veriti ABI). After completion of PCR amplification, 5 µL of each sample was electroporated by electrophoresis in 2% agarose gel (1st Base, Singapore) staining with FloroSafe DNA stain (1st Base, Singapore) for 45 min and 110 V in 1x Tris-acetate-EDTA (TAE) buffer. DNA bands were visualized by image analyzer (Syngene).

RESULTS AND DISCUSSION

EPIDEMIOLOGICAL DATA ANALYSIS

Out of 23 bacterial isolates listed in Table 1, 17 (73.9%) were *Klebsiella pneumoniae* and six isolates were *Escherichia coli* (26%). Seven isolates (41.2%) of the total 17 *K. pneumoniae* were determined to be ESBL producers using the automated VITEK-2 system and the phenotypic test disc diffusion test (PDCT). Four *K. pneumoniae* (23.5%) isolates were CRE strains and six (35.3%) isolates were susceptible strain. One *K. pneumoniae* ATCC 1705 was used as a quality control strain in this study. Majority of the isolates (10; 43.5%) were isolated from blood samples. Other specimens were pus (8, 34.78%), urine (3; 13.04%), and sputum (2; 8.7%), respectively.

Our study showed that six *K. pneumoniae* ESBL and four CRE strains were multidrug resistant (MDR). According to a recent review article on nano-antibiotics (Li et al. 2023), MDR can be defined as resistant to at least one agent from three or more antibiotic classes. The emergence of multidrug resistance (MDR) *K. pneumoniae* strains has been identified as a major global concern, particularly in the field of infectious disease. This is consistent with a 2017 World Health Organization report that identified ESBL-producing and carbapenem-resistant Enterobacteriaceae as crucial targets for novel antibiotics due to the rise of these extremely resistant strains (González et al. 2021).

ANTIMICROBIAL SUSCEPTIBILITY TESTING (AST)

Table 3 showed that in *K. pneumoniae* ESBL strains, the AST profiles showed resistance in all isolates (7; 100%) towards cefuroxime and cefotaxime, respectively, while six of the isolates (85.7%) were resistant to ciprofloxacin and cefuroxime IV only. In contrast, none of the ESBL isolates were resistant to carbapenems antibiotics such as ertapenem, imipenem and meropenem. On the other hand, the AST profiles of all four *K. pneumoniae* CRE strains showed resistance in almost all antibiotics (4; 100%), while only few showed susceptible to aminoglycosides such as amikacin (2; 50%) and gentamicin (3; 75%). Susceptible isolates of *K. pneumoniae* showed high susceptibility (6; 100%) towards most of the antibiotics especially amoxycillin-clavulanate, cefuroxime, cefuroxime IV and gentamicin. Besides, Table 4 also portrayed susceptibility (5; 83.3%) towards amoxycillin-clavulanate, ampicillin, cefuroxime, cefuroxime IV, gentamicin, and piperacillin-tazobactam among *E. coli* isolates.

In this study, we focused on determining the antimicrobial susceptibility profiles and molecular characterization of *K. pneumoniae* and *E. coli* bacterial isolates. ESBL-producing *K. pneumoniae* showed resistance pattern towards cefuroxime, cefotaxime and ciprofloxacin. These were consistent with research results from China (Zhang et al. 2016), India (Sarojamma & Ramakrishna 2011), and Portugal (Carvalho et al. 2021). Our findings showed that the *K. pneumoniae* resistance rate to cefuroxime was comparable to a study on *K. pneumoniae* ESBL strain causing community-onset infection in China, where an alarmingly high rate of resistance to cefuroxime (97.9%) was observed among 189 isolates (Zhang et al. 2016). This is consistent with another study in Kurnool, India, which found 48% cefotaxime resistance and 72% ciprofloxacin resistance among *K. pneumoniae* obtained from tertiary care hospital (Sarojamma & Ramakrishna 2011). Another study found 100% ciprofloxacin resistance among 12 ESBL-positive isolates in a Portuguese hospital (Carvalho et al. 2021). High resistance rate across regions may suggest the widespread and uncontrolled use of

TABLE 2. Sequence of the primer used and size of the PCR amplicons

	Primer Name	Primer Sequences (5'-3')	Amplicon Size (bp)	Annealing Temperature (T_m ºC)	References
β-lactamase Target Gene: TEM, SHV, OXA, CTXM-1, CTXM-2, CTXM-8, CTXM-9, OXA-48, CTXM-15, CTXM-14	TEM (F)	CATTTCCGTGTCGCCCTTATTC	800		(Copur Cicek et al. 2013)
	TEM (R)	CGTTCATCCATAGTTGCCTGAC			
	SHV (F)	AGCCGCTTGAGCAAATTAAAC	713		(Dallenne et al. 2010)
	SHV (R)	ATCCCGCAGATAAATCACCAC			
	OXA (F)	GGCACCAGATTCAACTTTCAAG	564	60	(Copur Cicek et al. 2013)
	OXA (R)	GACCCCAAGTTTCCTGTAAGTG			
	CTXM-1 (F)	TTAGGAARTGTGCCGCTGYA	688		(Copur Cicek et al. 2013)
	CTXM-1 (R)	CGATATCGTTGGTGGTRCCAT			
	CTXM-2 (F)	CGTTAACGGCACGATGAC	404		
	CTXM-2 (R)	CGATATCGTTGGTGGTRCCAT			
	CTXM9 (F)	TCAAGCCTGCCGATCTGGT	561		(Dallenne et al. 2010)
	CTXM9 (R)	TGATTCTCGCCGCTGAAG			
	CTXM8 (F)	AACRCRCAGACGCTCTAC	326	57	(Woodford, Fagan & Ellington 2006)
	CTXM8 (R)	TCGAGCCGGAASGTGTYAT			
	OXA-48 (F)	TTG GTG GCA TCG ATT ATC GG	744		(Queenan & Bush 2007)
	OXA-48 (R)	GAG CAC TTC TTT TGT GAT GGC			
	CTXM-15 (F)	CAC ACG TGG AAT TTA GGG ACT	996		(Muzaheed et al. 2021)
	CTXM-15 (R)	GCC GTC TAA GGC GAT ÁAA CA			
	CTXM-14 (F)	TAC CGC AGA TAA TAC GCA GGT G	355	62	(Chia et al. 2005)
	CTXM-14 (R)	CAG CGT AGG TTC AGT GCG ATC C			
CRE Target Gene: IMP, VIM, KPC, NDM	IMP (F)	TTG ACA CTC CAT TTA CDG	139	55	(Dallenne et al. 2010)
	IMP (R)	GAT YGA GAA TTA AGC CAC YCT			
	VIM (F)	GAT GGT GTT TGG TCG CAT A	390		(Ellington et al. 2007)
	VIM (R)	CGA ATG CGC AGC ACC AG			
	KPC (F)	GGC CGC CGT GCA ATA C	60	56	(Azimi et al. 2013)
	KPC (R)	GCC GCC CAA CTC CTT CA			
	NDM-1 (F)	GGT TTG GCG ATC TGG TTT TC	621	52	(Candan & Aksöz 2015)
	NDM-1 (R)	CGG AAT GGC TCA TCA CGA TC			
AmpC β-lactamase: MOX, ACC, FOX, DHA, EBC, CIT	MOX (F)	GCA ACA ACG ACA ATC CAT CCT	895	60	(Dallenne et al. 2010)
	MOX (R)	GGG ATA GGC GTA ACT CTC CCA A			
	ACC (F)	CAC CTC CAG CGA CTT GTT AC	346		
	ACC (R)	GTT AGC CAG CAT CAC GAT CC			
	FOX (F)	CTA CAG TGC GGG TGG TTT	162		
	FOX (R)	CTA TTT GCG GCC AGG TGA			
	DHA (F)	TGA TGG CAC AGC AGG ATA TTC	997		
	DHA (R)	GCT TTG ACT CTT TCG GTA TTC G			
	EBC (F)	CGG TAA AGC CGA TGT TGC G	683		
	EBC (R)	AGC CTA ACC CCT GAT ACA			
	CIT (F)	CGA AGA GGC AAT GAC CAG AC	538		
	CIT (R)	ACG GAC AGG GTT AGG ATA GY			

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Housekeeping Gene: KHE, rPOB	KHE (F)	TGA TTG CAT TCG CCA CTG G	438	60	(He et al. 2016)
	KHE (R)	GGT CAA CCC AAC GAT CCT G			
	rPOB (F)	GGC GAA ATG GCW GAG AAC CA	1000	50	
	rPOB (R)	GAG TCT TCG AAG TTG TAA CC			

F: Forward, R: Reserve, bp: base pair, β-lactamase: Beta-Lactamases, CRE: Carbapenem-resistant Enterobacterales

antimicrobials in clinical practice (Moya & Maicas 2020). This emphasized the need for cautious and strategic antibiotic use in healthcare settings to mitigate further escalation of resistance.

Furthermore, in this study, all CRE strains showed increased resistance to almost all antibiotics including carbapenem antibiotics such as ertapenem, imipenem and meropenem. US Centers for Disease Control and Prevention (Centers for Disease Control and Prevention 2019) has recognized CRE as a public health threat as continuous overuse of these antibiotics has resulted in the emergence of CRE strains (Morrill et al. 2015; Vock & Tschudin-Sutter 2019), which explains the possibility of higher resistance rate to carbapenem in our study. Carbapenem antibiotics have been regarded as the 'last-line' defense antibiotic in the treatment of multidrug-resistant bacterial infections, particularly in infection cause by extended spectrum β-lactamase (ESBL) enzyme (Centers for Disease Control and Prevention 2019). The mechanism of resistant may be due to the ability of carbapenem-resistant *K. pneumoniae* to produce carbapenamases enzyme with the ability to hydrolyze β-lactam antibiotics including carbapenem (Leavitt et al. 2009; Nordmann, Dortet & & Poirel 2012; Pitout, Nordmann & Poirel 2015; Wu et al. 2011) or the production of beta-lactamases (like AmpC) combined with alterations to the bacterial cell membrane (like mutations in porin) (Nordmann, Dortet & & Poirel 2012). This can be explained where any mutation happens in the central channel of porin proteins, which act as channels for the passage of molecules across the bacterial outer membrane, can have a significant impact by preventing the antibiotics from entering the bacterial cell, thus reducing their effectiveness.

MOLECULAR CHARACTERIZATION OF THE BACTERIAL ISOLATES

The results in Table 5 showed that all seven isolates of *K. pneumoniae* ESBL harboured at least one of the β-lactamase-related genes such as bla_{TEM} (1; 14.3%), bla_{SHV} (1; 14.3%), bla_{CTXM-1} (4; 57.14%), and $bla_{CTXM-14}$ (2; 28.6%). Only one isolate (14.3%) has both bla_{TEM} and bla_{CTXM-1} genes coexisted. Co-existence of bla_{TEM} and bla_{CTXM-1} genes were detected among all CRE isolates (4; 100%). As for *K. pneumoniae* susceptible isolates, almost half of the isolates (50%) and one (16.67%) isolate harboured bla_{SHV} and bla_{CTXM-9}, respectively. On the other hand, *K. pneumoniae CRE* were found to harboured bla_{NDM-1} gene only (4, 100%) (Figure 1). No AmpC β-lactamase was detected in all ESBL, CRE and *K. pneumoniae* susceptible isolates. For housekeeping genes, all 17 (70.8%) and 15 (62.5%) *K. pneumoniae* isolates detected *khe* and *rPOB* gene, respectively. As for *E. coli*, no carbapenamases, ESBLs or, AmpC β-lactamase and *khe* genes detected. Only 2 (33.33%) of *E. coli* isolates harboured rPOB gene. These findings were further illustrated in Figure 2, which visually summarized the prevalence of resistance genes among *K. pneumoniae* and *E. coli* isolates, consistent with the data presented in Table 5.

Concerning the molecular characteristics, our study showed that bla_{TEM} *and* bla_{CTXM} were the most predominant genes detected in both ESBL and CRE *K. pneumoniae* in HPUSM, Kelantan. Similarly, these findings are consistent with several other research studies across different regions such as Pakistan (Ejaz et al. 2021), Iran (Feizabadi et al. 2010) and Iraq (Pishtiwan & Khadija 2019), where high prevalent of β-lactam genes such as bla_{SHV} and bla_{CTXM} genes were harbored from *K. pneumoniae* isolates. Coexistence of bla_{SHV}, bla_{TEM} and bla_{CTXM} was reported in 34.7% of *K. pneumoniae* isolates (Feizabadi et al. 2010), which was in line with current findings where bla_{TEM} and bla_{CTXM} were found coexisted in 100% of CRE strains and 14.3% of ESBL strain. The occurrence of β-lactam genes (bla_{SHV}, bla_{TEM} and bla_{CTXM}) were also found in *Escherichia coli* (Ejaz et al. 2021; Islam et al. 2022; Pishtiwan & Khadija 2019; Zhang et al. 2021) and *Pseudomonas aeruginosa* (Peymani et al. 2017; Shalmashi et al. 2022), reinforces the widespread nature of this resistance mechanism across different bacterial species. Thus, the presence of beta-lactamase genes, which efficiently hydrolyze or break down penicillin, cephalosporin, or carbapenem present in the antibiotics, can be attributed to the decrease effectiveness of those antibiotics towards *K. pneumoniae* (Jiang et al. 2020; Wei et al. 2018).

Furthermore, the *K. pneumoniae* carbapenem resistant reported here was classified as carbapenemase-producing strains, or CP-CRE. This can be explained as major carbapenemase family Metallo β-lactamases (MBLs) NDM-1 was detected in all of the CRE producing strains. This finding was consistent with the other previously published studies in Malaysia (Lee et al. 2022) and Singapore (Marimuthu et al. 2017). In University of

TABLE 3. Antimicrobial susceptibility profiles of ESBL, CRE and susceptible isolates of *Klebsiella pneumoniae* based on AST results

Antibiotics	*K. pneumoniae* ESBL (*n*= 7)			*K. pneumoniae* CRE (*n*= 4)			*K. pneumoniae* susceptible (*n*= 6)		
	Susceptible N (%)	Intermediate N (%)	Resistant N (%)	Susceptible N (%)	Intermediate N (%)	Resistant N (%)	Susceptible N (%)	Intermediate N (%)	Resistant N (%)
Amikacin	3 (42.9)	0	1 (14.3)	2 (50)	0	0	5 (83.3)	0	0
Amoxycillin-clavulanate	4 (57.14)	1 (14.3)	1 (14.3)	0	0	4 (100)	6 (100)	0	0
Cefepime	3 (42.9)	0	4 (57.14)	0	0	4 (100)	5 (83.3)	0	0
Cefotaxime	0	0	7 (100)	0	0	4 (100)	5 (83.3)	0	0
Ceftazidime	1 (14.3)	1 (14.3)	5 (71.4)	0	0	4 (100)	5 (83.3)	0	0
Cefuroxime	0	0	7 (100)	0	0	4 (100)	6 (100)	0	0
Cefuroxime IV	0	0	6 (85.7)	0	0	4 (100)	6 (100)	0	0
Ciprofloxacin	0	1 (14.3)	6 (85.7)	0	0	4 (100)	5 (83.3)	0	0
Ertapenem	3 (42.9)	1 (14.3)	0	0	0	4 (100)	5 (83.3)	0	0
Gentamicin (10mcg)	3 (42.9)	0	4 (57.14)	3 (75)	0	1 (25)	6 (100)	0	0
Imipenem	4 (57.14)	0	0	0	0	4 (100)	5 (83.3)	0	0
Meropenem	4 (57.14)	0	0	0	0	4 (100)	5 (83.3)	0	0
Piperacillin-tazobactam	4 (57.14)	0	1 (14.3)	0	0	4 (100)	5 (83.3)	0	0
Trimethoprim-sulfamethoxazole	3 (42.9)	0	3 (42.9)	0	0	4 (100)	5 (83.3)	0	0

ESBL: Extended-Spectrum-Beta-Lactamases, CRE: Carbapenem-resistant Enterobacterales, AST: Antibiotic Susceptibility Test, mcg: microgram

TABLE 4. Antimicrobial susceptibility profiles of *Escherichia coli* isolates based on AST results

Antibiotics	*E. coli* (*n*= 6)		
	Susceptible* N (%)	Intermediate** N (%)	Resistant*** N (%)
Amikacin	2 (33.3)	0	0
Amoxycillin-clavulanate	5 (83.3)	1 (16.7)	0
Ampicillin	5 (83.3)	0	1 (16.7)
Cefepime	2 (33.3)	0	0
Cefotaxime	2 (33.3)	0	0
Ceftazidime	2 (33.3)	0	0
Cefuroxime	5 (83.3)	0	0
Cefuroxime IV	5 (83.3)	0	0
Ciprofloxacin	0	2 (33.3)	0
Ertapenem	2 (33.3)	0	0
Gentamicin (10 mcg)	5 (83.3)	0	0
Imipenem	2 (33.3)	0	0
Meropenem	2 (33.3)	0	0
Piperacillin-tazobactam	5 (83.3)	0	0
Trimethoprim-sulfamethoxazole	3 (50)	0	0

AST: Antibiotic Susceptibility Test; mcg: microgram

*"Susceptible" here refers to category where the isolate was inhibited by the usually achievable concentrations of antimicrobial agent when the recommended dosage was introduced, indicating likely clinical efficacy

**"Intermediate" here refers to a category defined by a breakpoint that includes isolates with MICs or zone diameters approached attainable blood or tissue levels and response rates were potentially lower compared to susceptible isolates

***"Resistant" here refers to isolates that was not inhibited by usually achievable concentrations of the antimicrobial agent under normal dosage regimens

TABLE 5. Molecular characteristics of *K. pneumoniae* and *E. coli* based on specific targeted primers

Primer Name	*K. pneumoniae* ESBL							*K. pneumoniae* CRE				*K. pneumoniae* susceptible								*E. coli*					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	ATCC
β-lactamase Target Gene																									
TEM	-	-	-	/	-	-	-	/	/	/	/	-	-	-	-	-	-	-	-	-	-	-	-		/
SHV	-	-	-	-	-	/	-	-	-	-	-	/	/	-	/	-	-	-	-	-	-	-	-		-
CTXM-1	/	/	-	/	-	-	/	/	/	/	/	-	-	-	-	-	-	-	-	-	-	-	-		-
CTXM-9	-	-	-	-	-	-	-	-	-	-	-	-	-	/	-	-	-	-	-	-	-	-	-		-
CTXM-14	-	-	/	-	/	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-
CRE Target Gene																									
KPC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		/
NDM-1	-	-	-	-	-	-	-	/	/	/	/	-	-	-	-	-	-	-	-	-	-	-	-		-
Housekeeping Gene																									
KHE	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-	-	-		/
rPOB	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	/	/	-	-	-	-		/

ESBL: Extended-Spectrum-Beta-Lactamases, CRE: Carbapenem-resistant Enterobacterales

'–' indicates gene absence; '/' indicates gene presence. Genes not shown in the table were absent in all isolates as detected by Polymerase Chain Reaction (PCR) method

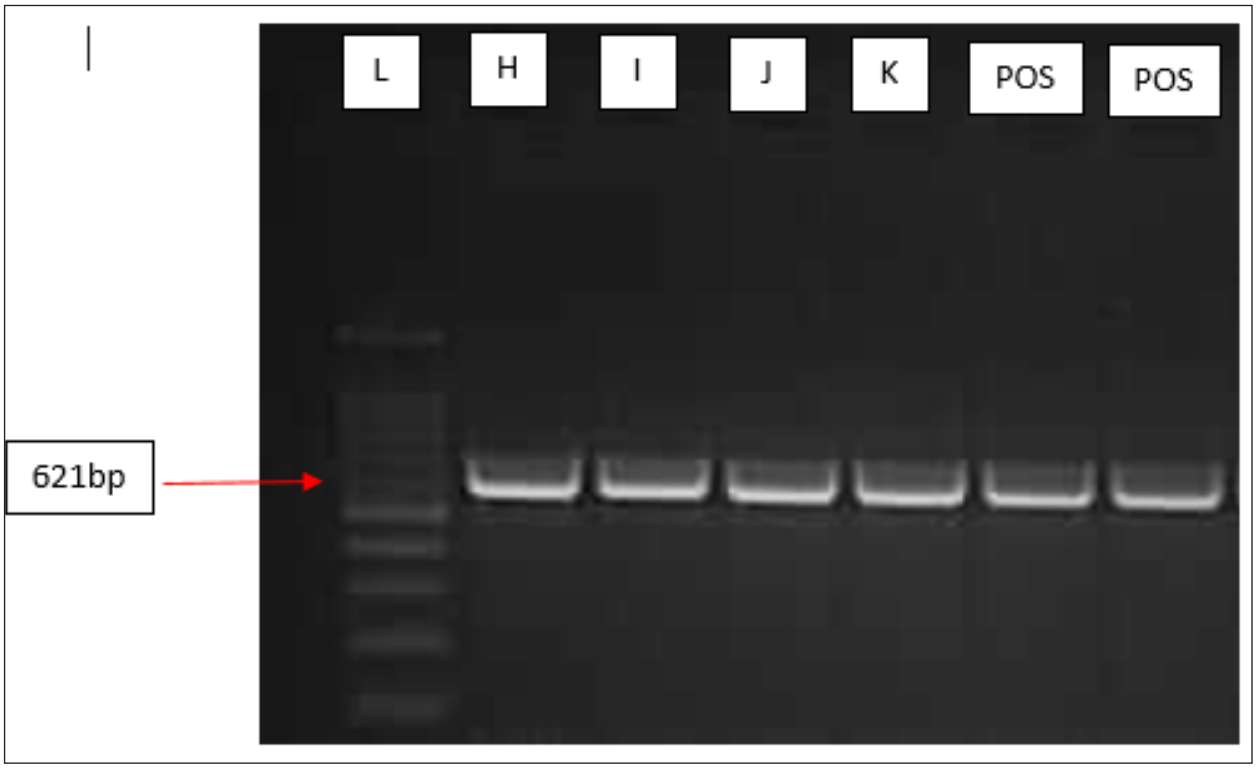

FIGURE 1. 2% agarose gel electrophoresis used for detection of bla_{NDM-1} in CRE producing *K. pneumoniae.* Lane L: 100 bp ladder (1st BASE, Singapore), Lane H, I, J, K: CRE-producing *K. pneumoniae* strains, Lane POS: NDM-1 positive *K. pneumoniae*

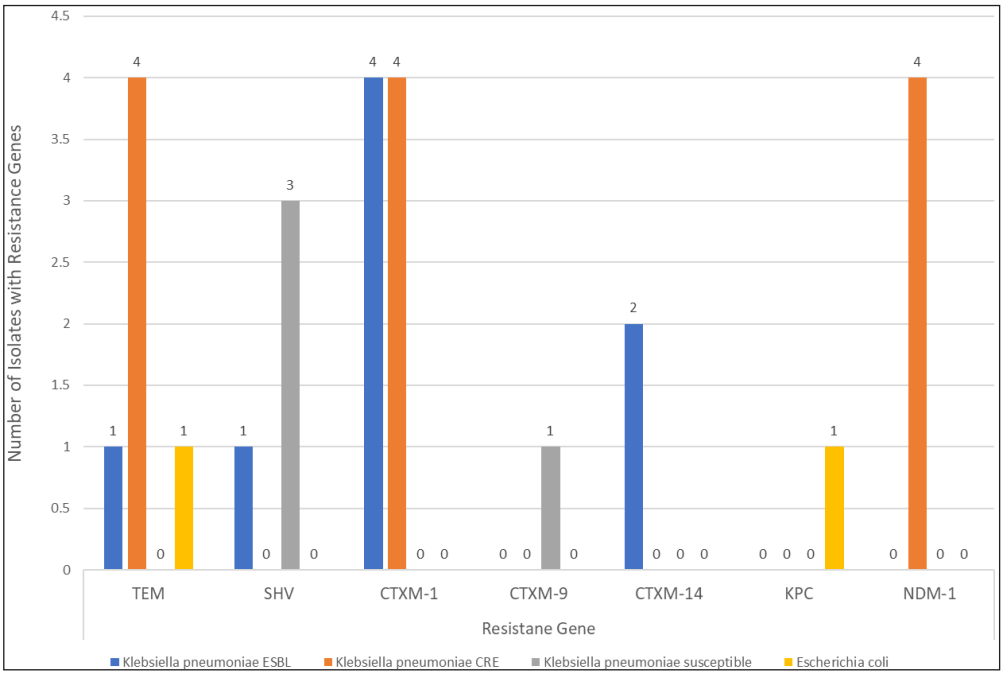

FIGURE 2. Prevalence of β-lactamase and carbapenemase resistance genes among *Klebsiella pneumoniae* and *Escherichia coli* isolates

Malaya Medical Centre (UMMC), Malaysia, they found out that most of their isolates from hospital setting were carbapenemase-producing *K. pneumoniae* (CP-CRE) with bla_{OXA-48} and bla_{NDM} as predominant genes (Lee et al. 2022). Moreover, a prospective study in Carbapenemase-Producing Enterobacteriaceae in Singapore (CaPES) shows that 71.4% (307/430) had CP-CRE and highly associated with chronic pulmonary disease and long-term hospital stay (Marimuthu et al. 2017). However, this is contrary with a study conducted in Taiwan where bla_{NDM} gene was not detected among 13.1% *K. pneumoniae* isolates (13/99 isolates) (Wu et al. 2022). Although a significant proportion of CP-CRE *K. pneumoniae* were found in several Southeast Asia (SEA) countries, there is still limited findings on the pathogenicity and clinical outcome of carbapenemase family belonging to MBLs in Malaysia.

Rapid identification of carbapenemase-producing strains especially involving MBLs mechanisms is crucial to help the clinicians to decide on possible antibiotic alternative such as polymixin B and cefiderocol to treat the patients as soon as possible. In parallel with these conventional therapeutic interventions, there is increasing recognition of the emerging paradigm-shifting strategies, particularly the application of bioinformatics and omics study in the understanding of antimicrobial resistance mechanisms. Whole genome sequencing (WGS), especially using long-read platforms, combined with antimicrobial resistance (AMR) prediction tools enables precise identification of resistance genes, mobile genetic elements and strain specific mutations. These insights are crucial in tracking the spread and evolution of resistance determinants.

Moreover, transcriptomic approaches such as RNA-sequencing technique allow for comprehensive profiling of gene expression in response to environmental stressors such as antibiotic exposure, nutrient starvation, or limited oxygen. RNA-sequencing offers the ability to determine which of the genes encoded in the DNA are turned on or off and providing a dynamic view of bacterial adaptation and resistance pathway. While genome analyses provide a complete picture of the genes present (and potentially expressed), only with high throughput transcriptomic profiling techniques can captures real-time expression patterns that can aid in pinpointing the intracellular targets and metabolic processes related to antibiotic mode of action and resistance (Zhang et al. 2025). Collectively, these integrative approaches highlight the urgent need for innovation in the fight against CP-CRE. Future efforts should prioritize coordinated surveillance and incorporation of omics-based data to guide precision antimicrobial strategies.

CONCLUSION

In conclusion, our study showed that bla_{TEM} and bla_{CTXM} genes were the most predominant genes detected in both *K. pneumoniae* ESBL and CRE isolates and bla_{NDM-1} genes was detected in *K. pneumoniae* CRE isolates which were in line with other findings worldwide. Target genes for β-lactamase and carbapenemase are linked to multiple

antibiotic resistance, making treatment more challenging. Understanding this link highlights the need for strategic antibiotic usage in healthcare settings by providing a deeper understanding of antibiotic resistance trends in multidrug resistant (MDR) organisms.

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