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RESEARCH ARTICLE

Molecular identification and antimicrobial susceptibility profiles of *Klebsiella pneumoniae* carrying Extended-Spectrum Beta-Lactamase (ESBL) associated genes in Kelantan, Malaysia

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ABSTRACT

Over the years, *Klebsiella pneumoniae* especially ESBL-producers type has become a global concern. Frequent reports on resistance reaction to a wide range of available antibiotics have resulted in uncontrolled hospital outbreaks. It is a therapeutic obstacle that must be overcome. This study was conducted to profile the microbiological characterization and antimicrobial resistance of ESBL-producing *K. pneumoniae*. Bacterial strains originated from human bodily fluid were subjected to a series of biochemical tests and disk-diffusion susceptibility test. Additionally, the molecular targeted ESBL-producer genes were established through PCR amplification to distinguish the β-lactamase type of the ESBL-producing *K. pneumoniae* isolates. All isolates were confirmed as ESBL-producing *K. pneumoniae* with biochemical characterization of acidic, yellow, rupture medium, positive Urease and Citrate test, and negative Indole test. Following the susceptibility test, the ESBL-producing *K. pneumoniae* isolates showed resistance to tested Ciprofloxacin, Ceftriaxone, Trimethoprim, Ampicillin, Nalidixic Acid, and Cephalothin. Lastly, the PCR amplification successfully identified at least one or more β-lactamase types in all isolates. The identified β-lactamase consists of TEM, OXA, SHV, CTX-M-1, and CTX-M-9. The presence of at least one or more of the β-lactamase gene in the *Klebsiella* strain should be taken seriously by the clinicians as the gene is capable to developed multidrug antibiotic resistance. Further and additional research need to be explored to fully understand the capabilities of ESBL-producer type bacteria.

1. INTRODUCTION

Nosocomial infection or hospital-acquired infection (HAI) is typically present in patients under medical care, which was absent during admission. Hospital-acquired pneumonia is known as one of the major causes of nosocomial infection (Khan *et al*. 2017). These infections are linked to the multidrug-resistant infection due to *Klebsiella pneumoniae*. *K. pneumo*niae is the most medically important *Klebsiella* species which contributes to the high nosocomial infections in healthcare environment. The infection is usually transmitted through gastrointestinal tract and unhygienic or contaminated hospital personnel, which can spread rapidly and capable of causing an outbreak. Hospital-acquired multidrug-resistant *K. pneumoniae* or ESBL-producing strain outbreak has been seen increasing in numbers over the past years (Ashurst and Dawson 2022).

ESBL or extended-spectrum beta-lactamase producers are a group of plasmid-mediated enzymes that become resistant to certain bactericidal antibiotics drug such as the penicillin, aztreonam, first, second, third and fourth generation cephalosporins in which cephalosporins belong to the beta-lactam group (Castanheira *et al*. 2021). The first described ESBL-producer related to *K. pneumoniae*, *Serratia marcescens* and *Escherichia coli* isolates were found in 1983 in the Europe (Knothe et al. 1983). The report highlighted that the resistance of all isolates to newer types of cephalosporin drug. Following that, the ESBL-producer related gene was next reported in United States in 1989. It was reported that the *K. pneumoniae* isolates exhibit resistance to ceftazidime and aztreonam due to the presence of TEM-type β-lactamase enzyme (Quinn *et al*. 1989). The emergence of infection since then has put the ESBL-producing *K. pneumoniae* as an important cause of nosocomial infection in the United State (McDanel *et al.* 2017) and Europe (Richelsen et al. 2020).

In Malaysia, the first case of ESBL-producing *K. pneumoniae* was reported in 2004 (Low *et al.* 2017). It was isolated from a blood culture of 42-year-old woman, which reported having carbapenem-resistant of *K. pneumoniae*. Association of ESBL-producing *K. pneumoniae* with nosocomial outbreak has been extensively studied over the

years. Previous study, Mobasseri *et al*. (2020) has reported on a nosocomial infection outbreak related to ESBL-producing *K. pneumoniae* isolated from a tertiary hospital in Malaysia. Continuous emergence of antibiotic resistance on microorganism specifically *K. pneumoniae* has contribute to the ongoing research related to the resistance aspect. The adaptability characteristics of the microorganism have made the antibiotic resistance to frequently occur when new antibiotic is created and administered. Diverse, complex and rapidly-evolving enzymes of ESBL-producing strain infection cause a major therapeutic challenge nowadays to treat hospital-acquired and community-based infections. Resistance resulted from beta lactamase to antibiotics through random mutation leads to difficulties to treat this ESBL infection (Rawat and Nair 2010a).

This study aims to determine the ESBL-producing *K. pneumoniae* through microbiological characteristics and antimicrobial resistant profiles as well as to implement molecular characterization of targeted ESBL-producing related genes. Therefore, additional and persistence research related to antibiotic resistance must be done for the advancement of knowledge. This available information will facilitate future treatment strategies

2. MATERIALS AND METHODS

2.1. Isolation and identification of bacterial strain

Five bacterial strains used in this study were randomly collected from archived sample storage at Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia (USM), Malaysia. The samples were stored in -80°C which originally retrieved from patient clinical fluid. Each strain was pipetted from glycerol stock and inoculated into 10 ml nutrient broth under sterile condition and was left overnight in incubator shaker at 37°C. Subsequently, after 18-hour incubation period, the bacterial broth was transformed onto Nutrient agar and MacConkey agar by applying a streaking technique using sterile wire loop. Following 18-hour incubation period at 37°C, single colony of each bacterial strain on nutrient agar was further subjected to biochemical tests namely Triple Sugar Iron test, Urease test, Citrate test, Indole test and Methyl Red test (Osman *et al*. 2020). Meanwhile, the colony growth on the McConkey agar was observed for its lactose metabolism characteristics to further classifying the bacteria as a gram-negative organism (Jung and Hoilat 2022a).

2.2. Antimicrobial susceptibility testing

The antibiotic susceptibility of bacteria was conducted through disk diffusion method using Mueller-Hilton (MH) agar plates (Bolouiri *et al*. 2016). Cultured bacteria colony from the Nutrient agar were selected and suspended overnight in Nutrient broth. These suspensions were left incubate at 37°C in shaking condition. Approximately 100 µl of bacterial suspension was inoculated onto the MH agar, which was then lawned using sterile swab covering all diameters. The concentration of all bacterial suspension was uniformly measured using OD600nm with measurement ranges from 1.5 to 2.0 nm. Then, the antibiotics disk was placed on the agar consisting of, Ciprofloxacin (5 µg), Ceftriaxone (30 µg), Trimethoprim (5 µg), Ampicillin (10 µg), Nalidixic Acid (30 µg) and Cephalothin (30 µg). Following 37°C overnight incubation, the presence of zone inhibition is recorded and measured. The diameter if inhibition zone is measured using ruler or caliper from the center of the disk to the point on the circumference, then multiply the measurement by 2 to determine the final value. The determination of antimicrobial susceptibility is based on the Clinical Laboratories and Standard Institute guidelines (CLSI 2018). The antimicrobial susceptibility testing was performed in three replicate testing.

2.3. Multiple antibiotics resistance (MAR) index analysis

The MAR Index was calculated by multiplying the number of antibiotics to which the isolate is resistant with the total number of antibiotics to which the isolate is exposed. MAR index value that is greater than 0.2 identified as high-risk source of infection in a region where the drugs are widely used (Amicosante *et al.* 2023)

2.4. DNA extraction

The extraction process for each bacterial bacteria strain was initiated by selecting a single colony from prepared agar plate and mixed it into the nutrient broth. Following the 37°C overnight incubation, approximately about 2 ml were aspirated for centrifugation process to obtain the pallet. Each bacteria pallet was subjected to DNA extraction using the QIAamp DNA mini-Kit (QIAGEN, USA) according to the manufacturer's protocol. The extraction process was completed by eluting the DNA product with TE buffer. The purity of the DNA was measured using Nanodrop spectrophotometer. Absorbance was recorded at wavelengths of 260 and 280 (A260 and A280, respectively) nm (Ghatak *et al.* 2013). The DNA was then stored at −20 °C until further analysis.

2.5. Molecular PCR amplification

The DNA product of each strain was subjected to molecular confirmation on ESBL-*K. pneumonia* through Polymerase Chain Reaction (PCR) method. The primer for the PCR reaction included forward primer (5'-GTTTGATCCTGGCTCAG-3') and reverse primer (5'-

GGTTACCTTGTTACGACTT-3') (Nima *et al.* 2020). Further PCR amplifications of ESBL-producers type by targeting seven specific primers that listed in Table 1.

Table 1: Primer sequence and annealing temperature (Tm (°C)) used for PCR amplification (Dos Santos *et al.* 2019).

Primer	Sequence		Tm (°C)
	Forward	Reverse	
TEM	5'-CATTTCCGTGTCGCCCTTATTC-3'	5'-CGTTCATCCATAGTTGCCTGAC-3'	60
OXA	5'-GGCACCAGATTCAACTTTCAAG-3'	5'-GACCCCAAGTTTCCTGTAAGTG-3'	60
SHV	5'-AGCCGCTTGAGCAAATTAAAC-3'	5'-ATCCCGCAGATAAATCACCAC-3'	60
CTXm–1	5'-TTAGGAARTGTGCCGCTGYA-3'	5'-CGATATCGTTGGTGGTRCCAT-3	60
CTXm–2	5'-CGTTAACGGCACGATGAC-3'	5'-CGATATCGTTGGTGGTRCCAT-3'	54
CTXm–9	5'-TCAAGCCTGCCGATCTGGT-3'	5'-TGATTCTCGCCGCTGAAG-3'	54
CTXm–8	5'-AACRCRCAGACGCTCTAC-3'	5'-TCGAGCCGGAASGTGTYAT-3'	57

Polymerase Chain Reaction (PCR) was performed using commercial kit, GoTaq® FlexiDNA Polymerase (Promega, USA) for all DNA samples. The 25 µL reaction mixture contained 1.0 mM MgCl2, 5 µL of 5X Green Flexi Buffer, 200 µM PCR nucleotide mix, 10 µM of each primer, 1.25 U of DNA polymerase and 0.5 µg of template DNA sample. The reaction involved initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing temperature range from 54 °C – 60 °C for 40 seconds and extension at 72 °C for 1 min followed by final extension at 72 °C for 2 min. Following amplification, PCR products were analyzed by 2% agarose gel electrophoresis in 1X TAE buffer (Promega, USA).

3. RESULT AND DISCUSSION

3.1 Isolation and identification of isolated bacteria

K. pneumoniae is considered as one of the clinically significant pathogens from *Klebsiella* spp. Since it was first identified, the *K. pneumoniae* has become a public concern due to its capability of causing variable diseases and its growing resistance to antibiotics (Effah *et al.* 2020). Recognition of isolated bacterial was commonly diagnosed based on culture characterization as well as biochemical tests. The proper identification of *K. pneumoniae* isolates in the clinical settings is crucial to avoid any misclassification which consequently leads to incorrect treatment plan.

In this study, five bacterial strains were individually cultured and grown in Nutrient broth. All nutrient broths exhibited changes in appearance after 18-hour of incubation from clear to cloudy and turbid. The identification of isolated bacteria were further cultivated on Nutrient agar and MacConkey agar. Following overnight incubation, observations on nutrient agar plate as shown in figure 1 revealed a greyish white color with circular and dome-shaped feature. Concurrently, the bacteria growth on MacConkey agar appeared to be moderately colonized with pink-red brick, mucoid with convex structure. Although nutrient agar is used solely for the purpose of growing bacterial strains, growth on MacConkey agar can differentiate each bacterial strain based on the physical appearance of the agar plate. These characteristic suggested that the isolated bacteria are *K. pneumonia*. The colony observation with pink and mucoid growth was in line with the characterization of *K. pneumonia* isolated from clinical and environment samples collected in Egypt (Al-Jader *et al.* 2022) This finding is consistent with another study in Ethiopia which also recorded the same growth characteristics on the MacConkey agar (Amare *et al.* 2022). The emerging of pink colonies with sticky, wet structure is resulted from the bacteria lactose fermenting ability in gram-negative species which produces acidic by-products, a polysaccharide capsule that lower the pH (Jun and Hoilat 2022b).

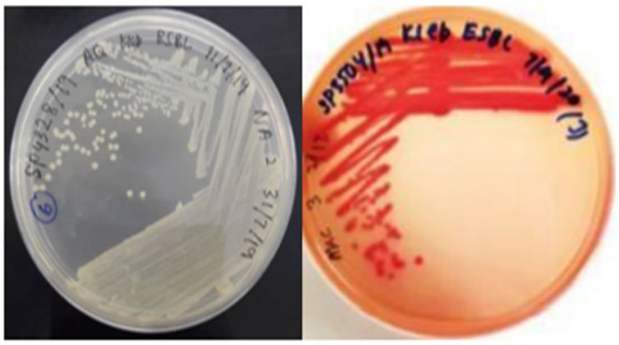

Figure 1: Appearance of the Nutrient agar and MacConkey agar after 18-hours incubation.

Further identification of *K. pneumoniae* using biochemical test is performed to confirm if the *K. pneumoniae* was correctly isolated. The outcomes of the biochemical tests namely Triple Sugar Iron test (TSI), Urease test, Citrate test, Indole test and Methyl-red test can be seen in Table 2. Changes in color and appearance were observed after 18-hour incubation under 37°C. Four of these biochemical tests showed the same pattern for all bacterial strains. The TSI test, resulted in an acidic reaction that yielded yellow color of the slant and butt of TSI agar with slight cracks and bubbles to the agar. This reaction indicated the presence of dextrose, lactose and sucrose fermentation with the formation of gases CO2 and O2. However, the blackening of agar is not recorded due to negative presence of the hydrogen sulphide (H2S) production (Aryal 2022a). Similarly, positive result could be

seen from the Urease and Citrate tests. The development of intense magenta, bright pink medium in the Urease test indicated the bacterial capability to hydrolyze urease enzyme into ammonia and carbon dioxide (Brink 2010). Meanwhile, the color change from green to intense blue in citrate test is resulted from the increase in the alkalinity of the agar therefore increasing the pH indicator. The positive citrate interpretation is an indication on the ability of an organism to utilize citrate as a source of energy in the development of bacterial successful growth (MacWilliams 2009).

Table 2: Characterization of biochemical test for five tested bacterial strains.

Sample Name	Biochemical Test							
	TSI							
	But	Slant	H2S	Gas	Urase	Citrate	Indole	Methyl Red
U7415	A	A	–	+	+	+	–	–
SP4328	A	A	–	+	+	+	–	+
BF6273	A	A	–	+	+	+	–	–
SP3504	A	A	–	+	+	+	–	–
HV4085	A	A	–	+	+	+	–	+

Besides that, the Indole test for all strains resulted in negative result, which can be seen when there is no formation of pink layer ring or no color change after the addition of appropriate reagent. Negative observation showed that the isolated bacteria do not have enzymes called 'typtophanase' that responsible in indole production (Aryal, 2022b). Concurrently, the final indicator, the Methyl-red test is the only biochemical test that does not yield the same pattern for all the bacterial strains. Three out of five bacterial strains showed negative result while the other two showed positive Methyl-red test with the formation of red ring layer at the surface of the medium subsequently adding the Methyl-red indicator solutions. Principally, the Methyl-red test purpose is to establish whether the bacteria have an ability to produce and maintain stable acid end products that comes from glucose fermentation process (McDevitt, 2009). Positive MR Test occurs when the substantial amounts of stable acid end product is produce by the bacteria whilst bacteria with negative MR Test indicated that the organism further metabolizes the initial fermentation product therefore decreases the acidity with no color changes on the medium (Shanmugaraj et al., 2021).

The biochemical profile of these isolated bacteria are in parallel with the culture growth observation that suggested *K. pneumonia* as identified strain with acid/acid reaction of TSI test, positive Urease and Citrate test, negative Indole test and variable MR test pattern. These biochemical observations are similar to the changes recorded from the isolation and identification of *K. pneumoniae* from patient clinical samples (Mustafa *et al.* 2023). Further finding on *K. pneumonia* biochemical test changes were in agreement with bacteria isolated form clinical samples (Sikarwar and Batra 2011; Rawy *et al.* 2020).

3.2 Antimicrobial susceptibility testing

The growing resistance of *Klebsiella pneumoniae* to all commonly used antimicrobial agents has become an alarming situation for all clinicians around the world (Patel *et al.* 2017; Patilaya *et al.* 2019). Table 3 tabulates the antimicrobial susceptibility result of all *K. pneumoniae* isolates. This study observed that *K. pneumoniae* showed resistance to all targeted antibiotics such as Ciprofloxacin, Ceftriaxone, Trimethoprim, Ampicillin, Nalidixic acid, and Cephalothin according to CLSI standard. A similar study has reported on the resistance effect of all six antibiotics towards *K. pneumoniae* (Garbati and Al-Godhair 2013; Alaali and Bin Thani 2020).

Table 3: The antimicrobial susceptibility results.

Antibiotics	Dose	Inhibition Zone Diameter (mm)	CLSI Diameter Guideline (mm)			R/I/S
			R	I	S	
Ciprofloxacin	5µg	10.00 ± 8.70	≤ 15	16 – 20	≥ 21	Resistant
Cefriaxone	30µg	8.60 ± 6.05	≤ 13	14 – 20	≥ 21	Resistant
Trimethoprim	5µg	11.60 ± 10.07	≤ 10	11 – 15	≥ 16	Intermediate / Resistant
Ampicilin	10µg	0 ± 0	≤ 13	14 – 16	≥ 17	Resistant
Nalidixic acid	30µg	6.40 ± 7.48	≤ 13	14 – 18	≥ 19	Resistant
Cephalothin	30µg	3.00 ± 5.30	≤ 14	15 – 17	≥ 18	Resistant

The resistance of more than one antibiotics suggested that the isolated *K. pneumoniae* bacterium is a multidrug-resistant strain. Occurrence of multidrug resistance in bacteria may be due to the accumulation of multiple coding of resistance gene within a single bacterium cell. Concurrently, the increase expression of gene that responsible for multidrug efflux pumps has also contribute to the increasing chance of multidrug resistance to happened in a bacterium (Domingues *et al*. 2023). The bacteria's ability to generate an enzyme contributing to deactivating antibiotics can cause the resistance effect of targeted antibiotics. The enzyme inhibits the concentration of the antibiotics, resulting in high risk of therapeutic failure (Helmy *et al*. 2023).

The MAR index is further calculated as an important tool to determine the origins and the dangerous level of the antibiotic-resistant bacteria (Reverter *et al.* 2020). The present research revealed the suggested *K. pneumonia* isolates have a MAR index of 0.83 which is greater than the preference indicator index. This value confirmed the presence of multidrug-resistant genes in the isolated bacteria, where each represents a single antibiotic resistance phenotype (Ejiofor *et*

al. 2016). Similar studies on MAR Index of *K. pneumonia* were reported to have the same value of greater than 0.2 (Afunwa *et al.* 2020; Ghenea *et al.* 2021; Okafor and Nwodo 2023).

3.3 Molecular identification of isolated bacteria

Further confirmation on suggested *K. pneu*monia isolates were molecularly amplified using 16S rRNA primers. As shown in Figure 2 (a) and (b), (L3) U7415, (L4) SP4328, (L5) BF6273, (L6) SP3504 and (L7) HV4085 represent ESBL-producing *K. pneumoniae* whilst (L1) ATCC2592 and (L2) ATCC1705 represent non-ESBL-producing *K. pneumoniae* and *E. coli* species respectively. The *E. coli* serves as a control strain. All the tested *K. pneumoniae* species, ESBL or non-ESBL at L2 line until L7 line were successfully amplified at a base-pair of 1450bp. Additionally, the L1 that represent *E. coli* species also showed amplification reaction with the presence of band with the same base pair. The universal gene such as the 16sRNA could be applied to examine the organism phylogenetic identification including that has not yet been cultivated. Our study employed the 27F1 and 1492R pair primer, the most commonly used primers for identification of bacterial genes, where the 27F1 is chosen at the spanning positions of 8 to 27 in *E. coli* rRNA coordinates (Wang *et al.* 2008). Meanwhile, the 1429R is chosen at the spanning positions of 1492 to 1507 which amplify nearly entire targeted genes (Wan Makhtar *et al.* 2020). Pairing and combination of these two-primer resulted with commonly binding site variant that cover most of the bacteria phyla. The pair primer-binding site sequences indicated that the 27F1 and 1492R amplification occurrences could be commonly observed dominantly at several phylogenetic group especially enteric bacteria (Frank *et al.* 2008).

The progression degree of antimicrobial resistance of *K. pneumoniae* is related to the type of extended-spectrum beta-lactamase (ESBL)-producer that is encoded in their gene. The most frequently found β-lactamase enzymes in clinical isolates are SHV-type ESBLs, TEM-type ESBLs, CTX-M-type β-lactamase, and OXA-type β-lactamases (Dallenne *et al.* 2010; Chookaew *et al.* 2012). All *K. pneumoniae* isolates undergo DNA extraction followed by PCR amplification using specific primers. Table 4 specifies the ESBL-producer type found in each *K. pneumoniae* isolates. From the table, each isolate has at least two or more ESBL-producer types that are encoded in their gene, which varied between TEM, OXA, SHV, CTX-M-1, and CTX-M-9. This finding is in agreement with previous ESBL associated gene study where more than one ESBL-producer can be detected in one isolate (Tissera and Lee 2013). The β-lactamase enzymes CTX-M-1 type from CTX-M group has been reported to be the most identified types among all CTX-M type (Rivoarilala *et al.* 2018). Meanwhile, the findings of this current study also indicate that CTX-M-2 and CTX-M-8 are not detected in any *K. pneumoniae* isolates. The presence of these ESBL associated genes has been previously reported to be commonly found in *Salmonella* species (Jure *et al.* 2010).

Each β-lactamase enzyme can be visually confirmed by calculating the band size which appears on the agarose gel after performing the PCR amplification (Figure 2 (b)). All targeted β-lactamase enzymes have their expected base-pair (bp) originated from the specific primers. The calculated base-pair for TEM gene is 800 bp, OXA gene is 564 bp, SHV gene is 731 bp, CTX-M-1 gene is 688 bp and lastly CTX-M-9 gene is 561 bp. For CTX-M-2 and CTX-M-8, the base-pair is not calculated due to unrecorded occurrence in this study. The presence of ESBL associated gene is often can be found located on large plasmids where it simultaneously carries the genes that result in resistances toward antibiotics (Rawat and Nair 2010b). ESBLs are a group of β-lactamase that has undergone amino acid substitution therefore increasing the affinity and the hydrolytic activity against classes of antibiotics. The synchronization between β-lactamase antibiotic resistance and the nature ability of *K. pneumonia* virulence genes could lead to the failure of treatment in related infection (Soltani *et al.* 2020).

Table 4: Molecular PCR-amplification with specific primer (ESBL-gene).

Sample Name	Primer / Gene Specific						
	TEM	OXA	SHV	CTX-M-1	CTX-M-2	CTX-M-9	CTX-M-8
U7415	+	+	+	+	–	–	–
SP4328	–	+	–	+	–	–	–
BF6273	–	–	+	+	–	–	–
SP3504	+	–	+	–	–	–	–
HV4085	–	+	–	–	–	+	–

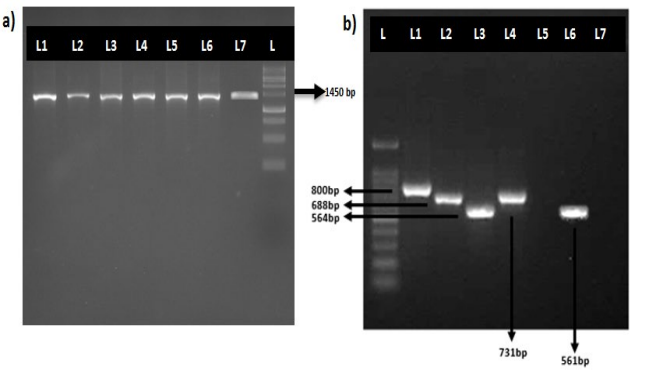

Figure 2: Polymerase Chain Reaction of DNA Amplification. (a) DNA gel electrophoresis after PCR amplification with 16S rRNA primer [(L1) ATCC 2592 (L2) ATCC 1705 (L3) U7415 (L4) SP4328 (L5) BF6273 (L6) SP 3504 (L7) HV 4085 (L) 1kb Ladder]; (b) DNA gel electrophoresis after PCR amplification with β-lactamase specific primers [(L) 100bp Ladder (L1) TEM (L2) CTXM-1 (L3) OXA (L4) SHV (L5) CTXM-2 (L6) CTXM-9 (L7) CTXM-8].

4. CONCLUSION

Through microbiological identification and biochemical characterization, all samples were successfully identified as *K. pneumoniae*. Additionally, the antimicrobial

test indicated that all identified *K. pneumoniae* showed resistance reaction to five antibiotics. Molecular identification of each *K. pneumoniae* on ESBL-producing gene showed that at least one type of gene is present in each strain. This study suggests that further research needs to be implemented to fully understand the mechanism of resistance of the strain to antibiotics.

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