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Whole-genome sequencing of extended spectrum beta lactamases (ESBLs)-producing *Klebsiella pneumoniae* (kp) isolates from selected hospitals in Malaysia

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Abstract
The resistance of ESBLs-producing Kp to various groups of antibiotics commonly used against infections they caused had become a global threat and required urgent attention. This study assessed the extended spectrum beta-lactamases (ESBLs)-producing *Klebsiella pneumoniae* isolates in terms of their genomic resistance. An analytical profile index (API) 20E kit was used to confirm a total of 100 clinical isolates of ESBL *Klebsiella pneumoniae*. The disc diffusion method was used to perform the antimicrobial susceptibility testing (AST), which was followed by the phenotypic detection of ESBLs. Six profiled representative ESBL positive strains were subjected to whole genome sequencing (WGS), multilocus sequence typing (MLST), and phylogenetic tree construction using the sequence data. The study showed that 46(46%) of the 100 isolates were positive for ESBL production and antibiotic susceptibility testing revealed significant resistance to β-lactam antibiotics including monobactam especially ampicillin/sulbactam (40%), cephalosporin groups (cefuroxime, cefotaxime, and ceftriaxone) stood at 51%, 49% and 48% respectively and aztreonam with 49%. The WGS analysis of the representative strains revealed genes encoding resistance to aminoglycoside (*StrA4*, *StrB1*, *aac(3')-IIa*, *aac(6')-1b*, *aac(6')1b-cr-1*, *aadA16*, *aph(3')-VIa* and *aadA15*), trimethoprim (*dfrA14* and *dfrA27*), sulphonamide (*sul1_11*, *sul2_2* and *sul2_3*), quinolone (*QnrB40-1*, *QnrB10*, *QnrS2*, *OqxA* and *OqxB*), tetracycline (tet(A)_4), fosfomycin (*fosA3*, *floR2* and *fosA7*), macrolid (*mph(A)_1*), rifampicin (*ARR-3*), β-lactam (*blaCTX-M-15_23*, *blaCTX-M-55*, *blaSHV-1_22*, *blaSHV11_18*, *blaSHV-11*, *blaSHV-1_1.1*, *blaSHV-11_3*, *blaSHV-11_19*, *blaTEM-1_1*, *blaTEM-1_5*, *blaOXA-51_10*, *blaOXA-30_1*, *blaNDM-1*, *blaLEN6*, *blaLEN8* and *blaLEN21* were detected. The MLST analysis revealed two novel sequence types of representative strains (2 with ST NF* and 12 with ST NF) and four other heterogeneous STs which include ST394, ST985, ST17 and ST11 while the phylogenetic tree of the strains showed closed clonal relationship and lineages with other reference isolates. In conclusion, the study's results showed a high prevalence of ESBL-producing Kp in the study area, and the representative strains' genomic contents demonstrated that ESBL-producing Kp in a clinical setting could serve as a reservoir for resistance genes and be the source of genetic transfer to other bacterial species. As a result, ongoing surveillance

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is required to monitor this endemic situation to prevent an epidemiological outbreak of *K. pneumoniae*- carrying ESBL.

Keywords Whole-genome, Sequencing, Extended spectrum β-lactamases, *Klebsiella pneumoniae*, Antibiogram and resistance genes

Introduction

Gram-negative bacteria are capable of producing enzymes known as Extended Spectrum Beta-Lactamases (ESBLs) that can confer resistance to some antibiotics such as cephalosporins (first, second, third, and fourth generation), aminopenicillins, as well as aztreonam but are inhibited by clavulanic acids [1]. ESBLs are chromosomal or plasmid-mediated enzymes that hydrolyze or inactivate beta-lactam antibiotics or in other words, are enzymes that hydrolyze a wide variety of beta-lactam antibiotics including oxymino-cephalosporins and aztreonam but inhibited by beta-lactam inhibitors like clavulanic acids, tazobactam, and sulbactam [2]. The appearance of ESBLs producers has complicated the treatment options and therefore poses a serious threat in the hospital setting. Usually seen in clinical settings, these enzymes are among the factors leading to antibiotic resistance and can result in reduced sensitivity or resistance to many antibiotics [3]. The ESBLs are mainly produced by *Klebsiella pneumoniae* and other Enterobacteriaceae families. Infections due to β-lactamases producing Klebsiella species are increasingly recognized in recent years thereby creating clinical concerns because few antibiotics are available as therapeutic options. *Klebsiella pneumoniae* is an integral part of the community and nosocomial infections pathogens particularly in immunocompromised patients [4].

Detection of these genes in ESBL-producing *K. pneumoniae* by molecular methods or typing and their sensitivity pattern can unveil valuable information about its epidemiology and help physicians for the reasonable treatment of infections [5]. Determination of TEM and SHV genes by molecular techniques in ESBL-producing bacteria and their pattern of antimicrobial resistance can supply useful data about their epidemiology and risk factors associated with these infections [6].

The major cause of community and health associates infections has been attributed to *Klebsiella pneumoniae* [7]. Some underlying diseases such as biliary diseases, malignancy, diabetes mellitus, cirrhosis, bacteremia, osteomas, urinary and infection of the biliary tract as well as alcoholism have been reported to impair the defenses of a person and therefore increase the risk of *Klebsiella pneumoniae* infections [8]. A nosocomial outbreak in Malaysia with an association of the $bla_{\mathrm{SHV-5}}$ in *Klebsiella pneumoniae* was reported [9]. There is limited information about genotypes and genomes of ESBL-producing Kp in Malaysia, even though Kp is gaining importance as a difficult to treat antibiotic resistant pathogen by [3].

Therefore, the main objective of the study is to characterize ESBL-producing *Klebsiella pneumoniae* isolates for antimicrobial resistance determinants and genetic backgrounds from the selected hospital in Malaysia.

Materials and methods

Bacterial strains

A total of 100 *Klebsiella pneumoniae* isolates (both 36 archives and 64 fresh isolates from different samples such as urine, blood, sputum, pus, gastric and tracheal aspirates) were collected from Hospital Sultan Abdul Aziz Shah (HSAAS) University Putra Malaysia and Hospital Pakar Sultanah Fatimah, Muar, Johor, from August 2021 to February 2022 and the sample size was determined based on the study [10] and was calculated using the formula of [11].

Sample preparation

The collected clinical samples were inoculated directly on MacConkey agar by streaking method and were incubated at 37°c for 24–48 h [12, 13]. The incubated plates were all observed for growth after twenty-four hours and those with growth were immediately sub-cultured on fresh blood agar plates while those with no visible growth were allowed for forty-eight hours. The plates without growth on the previous day were observed for growth after forty-eight hours and were sub-cultured.

Bacterial strains re-identification

The stock cultured were sub-cultured and the 24-hour sub-cultured colonies were picked for Gram staining.

Isolates verification

Isolates re-identification and confirmation using API-20E analytical system

All *K. pneumoniae* isolates were sub-cultured from stock cultures onto MacConkey (Oxoid, UK) media (lactose positive, mucoid, convex-shaped colony). All cultured plates were aerobically incubated overnight at 37°C. Further identification of *K. pneumoniae* was done by using API-20E analytical (BioMatrieux, France) system. The system consists of 20 substrates that were impregnated in plastic wells. The test was conducted according to the French Manufactured company (BioMatrieux) instruction.

Antibiotic susceptibility testing (AST)

All of the verified *Klebsiella pneumoniae* isolates were subjected to antibiotic susceptibility testing of various classes of antibiotics based on the Kirby Bauer disk diffusion method and followed CLSI guidelines [14]. All the 100 re-identified *Klebsiella pneumoniae* isolates (archived and fresh) were tested against 19 antibiotics (Amoxillin/clavulanic acid (20/10µg), Cefoperazone/sulbactam (75/30µg), Ampicillin/sulbactam (10/10µg), Piperacillin/tazobactam (100/10µg), Ceftazidime (30 µg), Cefotaxime (30 µg), Cefuroxime (30 µg), Ceftriaxone (30 µg), Cefepime (30 µg), Cefoperazone (75 µg), Piperacillin (100 µg), Meropenem (10 µg), Imipenem (10 µg), Amikacin (30 µg), Gentamicin (10 µg), Netilmicin (30 µg), Ciprofoxacin (5 µg), Trimethoprim/sulfamethoxazole (1.25/23.75 µg) and Aztreonam (30 µg). All antibiotics are Oxoid brand. All the stock cultured of the *Klebsiella pneumoniae* isolates were sub-cultured on MacConkey agar (prepared according to the manufacturer's instruction) plates and incubated at 37^0C for 24 h. After the incubation period, a colony of each isolate was taken using a sterile wooden swab stick and suspended in a bottle containing 3 ml of normal saline and their turbidity was ensured to match with 0.5 Mcfarland standard. They were inoculated on already prepared Mueller Hinton Agar (prepared according to the manufacturer's instruction) plates. A maximum of six antibiotic disks were picked using sterile forceps and placed on the surfaces of the inoculum plates with a distance in between the disks of 24 mm while a quality strain of *E. coli* ATCC 25922 was used as control. The inoculum plates with antibiotic disks were incubated for 16–18 h at 37^0C and the zones of inhibition were measured and compared to CLSI standard guidelines as sensitive (S), intermediate (I), and resistant (R) after the incubation period.

Detection of ESBLs producing isolates

Screening for ESBL production and confirmation

Screening and confirmatory tests for ESBLs were performed in line with CLSI guidelines. The screening was done using the disk diffusion method where *Klebsiella pneumoniae* isolates which showed resistance to 3rd generation antibiotics such as ceftazidime, cefotaxime and ceftriaxone including cefepime and monobactam (aztreonam) were taken as potential ESBLs producers and proceeded for confirmatory tests by disk combination method where ceftazidime in combination with clavulanic acid was used [15]. In confirmatory tests, the colonies of 24-hour MacConkey agar sub-cultured plates of all the isolates were suspended in sterile bottles containing 3 ml of normal saline and ensured an even mixture. Using sterile wooden swab sticks the suspension of the isolates which match 0.5 Mcfarland standard were inoculated into MHA (prepared according to the manufacturer's instruction) plates. Sterile forceps were used to pick and placed the antibiotic disks onto the surfaces of the inoculum plates and 20 mm distance was ensured between the disks while *Klebsiella pneumoniae* ATCC 700,603 was used as control, then a 5 mm increase in zones of inhibition of ceftazidime plus clavulanic acid were taken as positive for ESBL production. The tests procedure and interpretation followed CLSI guidelines.

Genomic detection of resistance genes, MLST and phylogenetic analysis

DNA extraction

The DNA of the positive isolates of ESBL-producing *Kp* were extracted using monarch purification genomic extraction kits (Biolabs, New England) following the manufacturer's instructions.

PCR genotyping of ESBL genes

Klebsiella pneumoniae ATCC 700,603 served as a positive reference for the analysis, and the standard PCR assay was adopted to find resistance genes in ESBL *Klebsiella pneumoniae* isolates, including the ESBL genes (SHV, TEM, and CTX-M-15). IDT (Integrated DNA Technologies, Coralville, Iowa, USA) created and provided the primers. Table 3.1 had a list of every primer. 12.5µL of MyTaq Red Mix Mastermix, 1µL of each primer, 5µL of gDNA, and 5.5µL of nuclease-free water were used to create the PCR mixture, which had a final volume of 25µL. The cycling conditions were as follows: three minutes of initial denaturation at 75^0C, thirty cycles of denaturation at 95^0C for one minute, thirty seconds of annealing at temperature (5^0C below primers TM), one minute of extension cycle at 72^0C, and five minutes of final extension cycle at 72^0C.

The PCR products were visualized by electrophoresis in 1% agarose gels stained with Gelstar nucleic acid gel stain (Lonza brand, USA).

Whole genome sequencing (WGS)

A total of six representative ESBL *K. pneumoniae* isolates were used for whole genome sequencing (WGS) after isolate profiling was done among the isolates to find clusters (Table 3.2).

The extracted gDNA of those isolates stored at -30^0C was used after the necessary quality control assessment was performed as shown in Table 3.2. The method of [16] was adopted.

Library preparation and whole generation sequencing (WGS)

Using the Nextera XT DNA library sample preparation kit (Illumina, San Diego, CA USA), multiplexed paired-end libraries (2 × 300 bp) were used to prepare the library. The Nextera XT kit's transposon was used to prepare, segment, and tag the gDNA of the representative

isolates. Prior to performing a 12-cycle PCR reaction to amplify the DNA fragments and add primers and indices for dual-indexed sequencing of pooled libraries, unique adapters were compiled for each sample for labelling. The samples were normalised, pooled, and then subjected to 300-base paired-end read sequencing on the Illumina Miseq platform with 100x coverage. The manufacturer's instructions were followed for all setup and sequencing. Using software BBDuk (BBTools version 36) with a source of https://jgi.doe.gov/data-and-tools/bbtools/, the generated reads were quality trimmed. Strict observance was maintained when trimming reads below the threshold value (trimq) of 30, contaminant sequences (ref) of phix.fa, and discarding reads with length below the threshold value (minle) of 50. De novo assembly was carried out using SRST 2 version 1.1.0 and SPAdes version 3.9.0 to nullify any gaps.

Genome analysis
Prokka (Rapid prokaryotic genome annotation) version 1.11.1 pipeline server (https://www.ncbi.nlm.nih.gov/genome/annotation-prok/) received the assembled contigs for annotation [17].

Resistance to antibiotics
By utilising ResFinder, VirulenceFinder, and PlasmidFinder, respectively, to evaluate antimicrobial resistance genes, virulence factors, and plasmid replicons, the resistome of bacteria was obtained. A minimum match length of 80% and identification criteria of 98% were used in the analysis. Aminoglycosides, trimethoprim, sulphonamides, quinolones, tetracycline, fosfomycin, macrolides, rifampicin, and B-lactams were the antibiotic families evaluated.

MLST for sequence typing of the isolates
Seven housekeeping genes (gapA, infB, mdh, pgi, phoE, rpoB, and tonB) were used in multilocus sequence typing (MLST) analysis to assign sequence types (STs). In silico MLST analyses were conducted using these genes through bionumerics softwere, and MLST assignment of ESBL-producing *Kp* isolates was carried out using WGS data [17, 18].

Phylogenetic analysis
The evolutionary history was inferred by using the Maximum Likelihood method and Tamura 3-parameter model [19]. The tree with the highest log likelihood (-5477.33) was shown. The percentage of trees in which the associated taxa clustered together was shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura 3 parameter model, and then selecting the topology with superior log likelihood value. The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 0.00% sites). This analysis involved 34 nucleotide sequences. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 356 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [20, 21].

Table 1 ESBLs prevalence in both fresh and archived clinical isolates of *K. pneumoniae*

Type of Isolates	Number of ESBLs	Percentage (%)
Fresh clinical isolates	22	48
Archive clinical isolates	24	52
Total	46	100

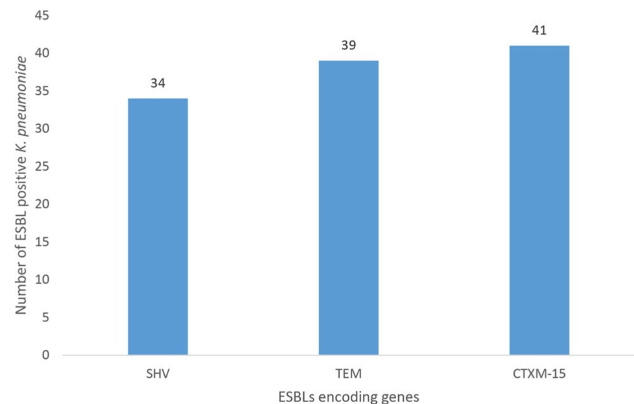

Fig. 1 Distribution of *bla*SHV, *bla*TEM and *bla*CTXM-15 on ESBL *K. pneumoniae*

Results
The result of the overall antibiogram pattern of one hundred (100) *K. pneumoniae* isolates which showed wide variation of susceptibility, resistance and intermediate responses against different types of antibiotics used were presented in Table 1 below:

Table 1 presented the result of ESBL prevalence in both fresh and archived clinical isolates of *K. pneumoniae* in which archived clinical isolates yielded the highest ESBL prevalence of *K. pneumoniae* isolates (24 (52%)).

The presence of different ESBL encoding genes such as *bla*SHV, *bla*TEM, and *bla*CTXM-15 was investigated among the positive ESBL isolates of both fresh and archived samples, and results obtained showed that CTXM-15 (41) was the dominant ESBL gene as displayed in Fig. 1 below:

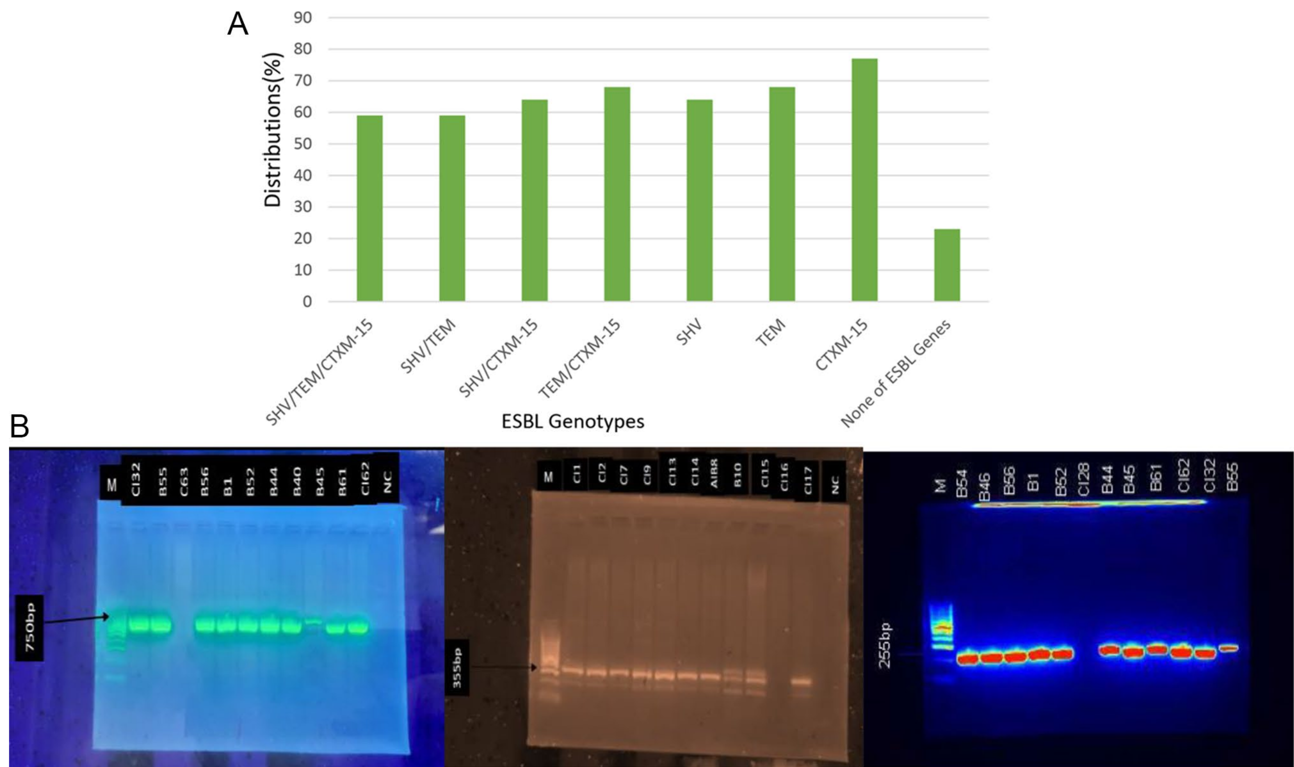

Fig. 2 **(A)** Genotypic profiles of ESBL associated genes in fresh clinical isolates of ESBL-producing *K. pneumonia*. (**B**) PCR detection of bla_{TEM} gene, bla_{SHV} and $bla_{CTX-M-15}$

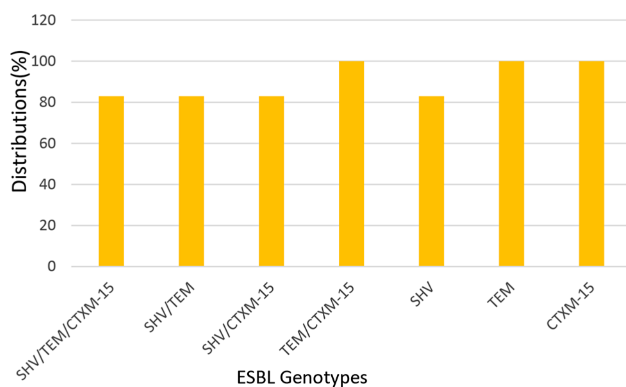

Fig. 3 Genotypic profiles of ESBL-associated genes in archived clinical isolates of ESBL-producing *K. pneumoniae*

Genotypic profile of ESBL encoding genes in fresh clinical ESBL-producing *Klebsiella pneumoniae*

The genotypic profiles of the ESBL encoding genes were studied in fresh clinical isolates of *Klebsiella pneumoniae* and the result obtained showed that CTXM-15 had the highest occurrence of 78% followed by TEM and TEM/CTXM-15 with 68% each and none of the ESBL genes were found in some isolates as shown in Fig. 2.

Genotypic profile of ESBL encoding genes in archived clinical ESBL-producing *Klebsiella pneumoniae*

The genotypic profile of ESBL encoding genes associated with archived clinical isolates of *Klebsiella pneumoniae* were investigated and the results obtained showed that CTXM-15, TEM and TEM/CTXM-15 were the most dominant ESBL genes as shown in Fig. 3.

The antibiotic resistance genes in ESBL *K. pneumoniae* were surveyed using whole genome sequencing method and genes resistance to various antibiotic classes (specifically 9 groups) were detected and presented in Table 2.

The summaries of both phenotypic characteristics such as cell morphology and gram status of the isolates as well as genotypic characteristics such as plasmid replicons and MLST of ESBL-producing *K. pneumoniae* were presented in Table 3.

The MLST result of the representative ESBL *K. pneumoniae* revealed four heterogenous variations and two novel sequence types which were shown in Fig. 4.

The result of evolutionary linages based on core genes of the six representative strains of ESBL producing *K. pneumoniae* revealed wide clonal relationship and lineages with other international strains as showed in Fig. 5.

Table 2 Antibiotic resistance genes detected in ESBL-producing *kp* isolates

Isolates	Isolates sources	Aminoglycosides	Trimethoprim	Sulphonamides	Quinolones	Tetracycline	Fosfomycin	Macrolides	Rifampicin	B-lactam
1	Fresh	strA4, strB1	dfrA14*	sul2_2	QnrB40-1*?, OqxA*, OqxB*	tet(A)_4	fosA3*	-	-	blaCTX-M-15_23, b; aLEN6*, blaSHV-1_22*?, blaTEM-1_1*
12	Fresh	strA4*, strB1*	dfrA14*	sul2_2	QnrS2*, oqxA*, oqxB*	tet(A)_4*	floR2*?, fosA7*	-	-	blaCTX-M-55, blaLEN6*, blaSHV-11_18*, bla-TEM-1_1* blaCTX-M-15_23, blaLEN8*, blaSHV-11
16	Fresh	aac(3)-IIa*, aac(6')-Ib,	-	-	QnrB10*?, oqxA*, oqxB*	tet(A)_4	fosA3*?	mph(A)_1	-	blaCTX-M-15_23, blaLEN8*, blaSHV-11
2	Fresh	strA4, strB1	dfrA14*	sul2_2	QnrB40-1*?, oqxA*, OqxB*	tet(A)_4	fosA3*	-	-	blaCTX-M-15_23, blaOXA-51_10, blaSHV-1_1.1, blaTEM-1_1
6	Fresh	aac(6')Ib-cr-1, aadA16*, aph(3)-Via*	dfrA27	sul1_11*?	QnrS2*, oqxA*, oqxB*	-	fosA3*	mph(A)_1	ARR-3	blaCTX-M-15_23, blaLEN6*, blaNDM-1, blaSHV-11_3, blaTEM-1_5
B13	Archived	aac(3)-IIa*, aac(6')Ib-cr-1, aadA15*?, strA4, strB1	dfrA14*	sul2_3	QnrB40-1*?, oqxA*, oqxB*	tet(A)_4	fosA3*	mph(A)_1	-	blaCTX-M-15_23, blaLEN21*, blaOXA-30_1, blaSHV-11_19*, blaTEM-1_1

Note: Only genes detected with > 90% coverage are reported

Key: - = not detected,

(?) is used to indicate uncertainty in alleles called

(*) is used to indicate imprecise matches in alleles called

Table 3 Summary of phenotypic and genotypic characteristics of ESBL-producing *kp* isolates

Isolates	Cell morphology	Gram Status	Hospital Name	Sample types	Samples source	Plasmid replicons	MLST
1	Rod-shaped	-ve	HSAAS	Fresh Clinical Sample	Sputum	FIBK_1_Kpn3_JN233704_92*	985
12	Rod-shaped	-ve	HSAAS	Fresh Clinical Sample	Blood	FIAHI1_1_HI1_AF250878_80*, FIIK_2_CP000966_pKp91_67*, FII_1_pKP91_CP000966_17*?,	NF
16	Rod-shaped	-ve	HSAAS	Fresh Clinical Sample	Urine	ColRNAI_1_DQ298019, FIAHI1_1_HI1_AF250878_80*, FIBK_1_Kpn3_JN233704_92, _FIIK_1_CP000648*, FII_1_pKP91_CP000966_17*, R 1 DQ449578	11
2	Rod-shaped	-ve	HSAAS	Fresh Clinical Sample	Sputum	FIBK_1_Kpn3_JN233704_92*,	NF*
6	Rod-shapedq	-ve	HSAAS	Fresh Clinical Sample	Urine	ColRNAI_1_DQ298019*, _FIIK_1_CP000648*, FII_1_pKP91_CP000966_17*?	17
B13	Rod-shaped	-ve	HPSF	Archived Clinical Sample	Urine	FIBK_1_Kpn3_JN233704_92*, FIBpQil_1_pQil_JN233705_318, _FIIK_1_CP000648*, FII_1_pKP91_CP000966_17*, _Q1_1_HE654726	394

Key: ID = Identity, (?) is used to indicate uncertainty in alleles called, (*) is used to indicate imprecise matches in alleles called, "NF" is used to indicate allele combinations not found in the MLST database, HSAAS = Hospital Sultan Abdul Aziz Shah, HPSF = Hospital Pakar Sultanah Fatimah, MLST = Multilocus Sequence Typing, - = not available, -ve = negative

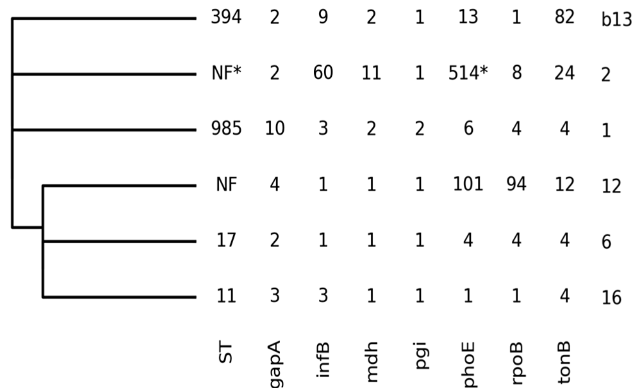

Fig. 4 MLST dendogram of the Representative Strains of ESBL-producing *Kp*

Discussion

Antibiogram pattern amongst pathogenic bacteria may extensively vary from country to country and within the same country from time to time or over a period of time [22]. The percentages of resistance to beta-lactam/beta-lactamase observed from this study were 40%, 24%, 23%, and 20% for Ampicillin/sulbactam, Amoxicillin/clavulanate, Piperacillin/tazobactam and cefoperazole/sulbactam with 7%, 14%, 15% and 23% intermediate rate, respectively. The percentages of resistance rates to cephalosporin groups were 51%, 49%, 48%, 47%, 39%,56%, and 31% for cefuroxime, cefotaxime, ceftriaxone, cefoperazone, ceftazidime, piperacillin, and cefepime, respectively while percentages of resistance to monobactam (Aztreonam) and aminoglycosides (Amikacin, gentamicin and Netilmicin) were 49%, 1%, 24% and 9%, respectively. The percentages of resistance to other group of antibiotics commonly used in clinical settings (ciprofloxacin and sulfamethoxazole/trimethoprim) were 33% and 21%, respectively. However, the study revealed high susceptibility of the isolates to carbapenem group (Meropenem and Imipenem) with 98% and 94% rates, respectively (Table 4).

The findings obtained from this study are higher than the reports of resistance rates of 5%, 21%, 5%, 15%, 21%, and 18% for Amoxicillin/clavulanate, Aztreonam, cefepime, cefoperazone, ceftazidime, and ceftriaxone, respectively by [4] and that of [1] with 83%, 31.9%, 27.8%, 27.8%, 25.7%, 19.5%, 11.3%, 8.2% and 7.2% for Amikacin, cefotaxime, cefoperazone, aztreonam, ceftazidime, amoxicillin/clavulanate, gentamicin, sulbactam/cefoperazole, and ciprofloxacin respectively. However, the results obtained from this study were lower than the percentages of antibiotics resistance rates reported by [23] and [3]. This could be due to different locations of the hospitals. The 40% resistance rate to beta-lactam/beta-lactamase (ampicillin/sulbactam) is in disagreement with the report of [6] that observed 46.67% sensitivity rate in beta-lactam/beta-lactamase (Piperacillin/tazobactam). Nevertheless, the findings from this study were similar to the report of high susceptibility of ESBL-producing *K. pneumoniae* to imipenem, meropenem, and amikacin in Indonesia by [24]. The findings revealed from this study

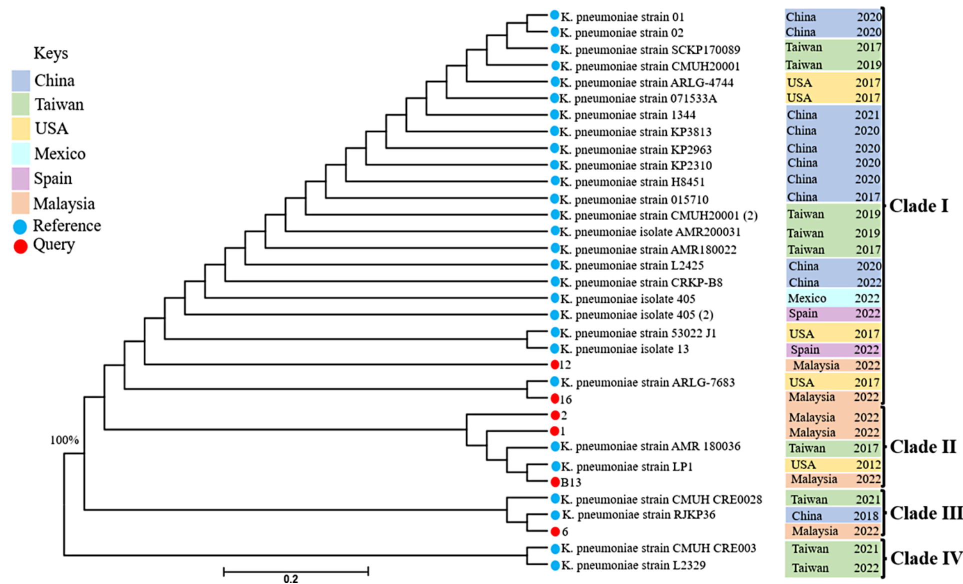

Fig. 5 Evolutionary lineages based on core genes of the six representative strains of ESBL-producing *Kp* and other international reference strains

Table 4 Antibiogram Pattern of 19 antibiotics tested on *K. pneumoniae* isolates

Antibiotics	Number of Isolates (%)		
	Susceptible [*n*(%)]	Intermediate [*n*(%)]	Resistance [*n*(%)]
Amikacin	95(95)	4(4)	1(1)
Ampicillin/Sulbactam	53(53)	7(7)	40(40)
Amoxicillin/Clavulanate	62(62)	14(14)	24(24)
Cefuroxime	24(24)	25(25)	51(51)
Cefotaxime	49(49)	2(2)	49(49)
Ceftriazone	52(52)	0(0)	48(48)
Cefoperazone	51(51)	2(2)	47(47)
Ceftazidime	54(54)	7(7)	39(39)
Cefepeme	54(54)	15(15)	31(31)
Aztreonam	50(50)	1(1)	49(49)
Cefoperazole/sulbactam	57(57)	23(23)	20(20)
Piperacillin/Tazobactam	62(62)	15(15)	23(23)
Sulfamethoxazole/Trimethoprin	24(52)	1(2)	21(46)
Imipenem	94(94)	4(4)	2(2)
Meropenem	98(98)	1(1)	1(1)
Gentamicin	76(76)	0(0)	24(24)
Netilmicin	86(86)	5(5)	9(9)
Ciprofloxacin	53(53)	14(14)	33(33)
Piperacillin	40(40)	4(4)	56(56)

in the case of high susceptibility to carbapenems were in concordance to the studies of [25] and various studies reported from India [6, 26, 27]. This report was also in agreement with the reports of Sid Ahmed et al., 2016 from Qatar and [26] from India on highest susceptibility to meropenem (100%). The results were also in line with [6] and [28] with the reports that all Klebsiella isolates were sensitive to imipenem and meropenem. Therefore, carbapenems can be considered as for the treatment of infections caused by ESBL-producing *Klebsiella pneumoniae*. The excessive and over use of cephalosporin antibiotics lead to the emergence of resistance and therefore shifting to alternative aminoglycosides therapy [29]. These agents that showed high resistance against ESBL-producing Kp isolates were due to the presences of resistances genes as seen in some of the representative strains (Table 2) and concluded to have limited capacity in treating infections caused by ESBL-producing Kp.

Based on the screening for ESBL production of the isolates (both fresh and archived) were first subjected to antibiotic susceptibility testing by disk diffusion method as reported by [14], and then 46/100 (46%) isolates out

of which 22/46 (48%) fresh and 24/46 (52%) archived isolates as shown in Table 1 were phenotypically confirmed as ESBL producers by disk combination method [14]. 46% of overall confirmed ESBL-producing isolates are highly alarming in the study area which simply implies that the residents of the area are at high risk of infections caused by ESBL-producing Kp or possibly ESBL-producing Kp outbreak. The high prevalence of the disease causative organism might be a result of hospital overstays, age, high immigrants rate etc. The results obtained from this present study on the prevalence of ESBL producing isolates (46%) was higher than that from other studies of [30] and [31], they reported ESBL prevalence of 19.8% and 24.56% respectively. The results of this study was also higher than the report of [32, 33] and [34]. However, the results obtained from the present study is in concordance with the report of [6, 35]. These results were also in agreement to the report of 43.5% ESBL prevalence in Iran [36], and also in line with the reports of [37] and [38].

The rising of ESBL production in species of bacteria varies greatly worldwide and trend of ESBL genes changes from time to time [39]. The prevalence of ESBL genes vary from one geographical region to other, TEM type was found to be prevalent in China among ESBL-producing *E.* coli, followed by SHV and CTX-M and similarly, SHV was predominant in Canada followed by TEM and CTX-M, while in India, over all other ESBL genes, CTX-M has been reported to be the most widespread [25]. The ESBL encoding genes screened for were three (bla_{SHV}, bla_{TEM}, and $bla_{\mathrm{CTX-M-15}}$), and results obtained from both isolates combined revealed 74% (34/46) bla_{SHV} prevalence, 85% (39/46) bla_{TEM} prevalence and 89% (41/46) $bla_{\mathrm{CTX-M-15}}$ prevalence while 11% (5/46) none of the encoding genes prevalence were detected in all the 46 isolates of both fresh and archived clinical *Klebsiella pneumoniae*(Fig. 1**)**. This may be due to the method used or the 5 isolates may be co-producing other β-lactamase enzymes such as Carbapenemase-producing *K. pneumoniae* (KPC) or Metallo-producing β-lactamase (MBL) and AmpC β-lactama and this could be a limitation to the study and other limitations of the includes, presence of more than one ESBL target genes in some isolates, non-detection of AmpC and carbapenemase ESBL production in the ESBL-producing isolates of *K. pneumoniae* and conduction of WGS on only six (6) representative strains due to financial constraints. In addition, all three ESBL encoding genes (SHV, TEM, CTX-M-15 and SHV/TEM encoding genes were detected in 72% (33/46) each of the confirmed isolates while 85% (39/46) encoding genes of TEM/CTX-M-15 was detected in the confirmed isolates of *Klebsiella pneumoniae.* On the basis of 24 confirmed archived isolates, the results obtained revealed that both $bla_{\mathrm{CTX-M-15}}$ and bla_{TEM} genes are the highest occurring encoding ESBL genes in the isolates with prevalence rates of 100% (24/24) each and bla_{SHV} with a least prevalence rate of 83% (20/24). In addition, there are harboring of encoding genes detected in the confirmed archived clinical isolates of *Klebsiella pneumoniae* where all the three encoding genes (SHV/TEM/CTX-M-15), SHV/TEM, and SHV/CTX-M-15 ESBL encoding genes were detected in 83% each of the isolates however, 100%(24/24) archived clinical isolates of *Klebsiella pneumoniae* harbor ESBL encoding genes of TEM/CTX-M-15 (Fig. 3**)**. The combinations of ESBL-genes or ESBL-producing Kp harbouring multiple genes observed in this study confers more resistance to other antibiotics such as gentamicin, ciprofloxacin and trimethoprim-sulfamethoxazole. This might be due to the fact that the congnitive plasmids harbouring ESBL genes, also often harbouring other antimicrobial resistance genes. This in line with the report of [40]. The results of ESBL genes combination revealed in this study is agreement with the reports of [41–43]. The prevalence of beta-lactamase encoding genes was also detected in 22 confirmed fresh clinical isolates of *Klebsiella pneumoniae* in which the results obtained showed that bla_{SHV} had the least prevalence of 64% (14/22) while $bla_{\mathrm{CTX-M-15}}$ had the highest prevalence of 77% (17/22) and followed by bla_{TEM} with the prevalence of 68% (15/22) in 22 confirmed samples. Furthermore, all three ESBL encoding genes (SHV, TEM, and CTX-M-15) and TEM/SHV encoding ESBL genes were detected in 59% (13/22) each of the positive samples, 64% (14/22) of the samples contained SHV/CTX-M-15 ESBL encoding genes and 68% (15/22) of the fresh clinical isolates of *Klebsiella pneumoniae* harbor both TEM/CTX-M-15 encoding genes (Fig. 2**)**. High mortality in developing nations has been linked to the rising incidence of ESBL-producing Kp [44]. In this investigation, however, a 46% frequency was seen in both fresh and stored clinical samples. This is in line with a report by [45] that found that in the public healthcare system in the South African provinces of KwaZulu-Natal, the Free State Gauteng, Limpopo, and the Western Cape, there was a 68.9% prevalence of ESBL-producing Kp bloodstream infection [46]. also observed a 32.43% prevalence of ESBL-producing Kp from faecal carriage in healthy patients admitted to tertiary hospitals. The results, however, were greater than those reported by [47], which indicated that among healthy residents of nursing homes in Lebanon, 9.7% of ESBL-producing Kp was detected through faecal carriage.

The genomic characterization of diverse resistance determinants presents in ESBL-producing Kp isolates was investigated in healthcare setting. The results obtained revealed high level of resistance as different resistance genes were detected (Table 3). Isolate B13 ST 394 harboured the highest resistance followed by isolate 6 ST17, then isolate 12 ST NF, while isolate 1 and 12 with ST 985 and ST NF* respectively harboured equal

resistance genes but isolate 16 ST 11 harboured the least resistance genes as shown in Table 3. These resistance genes obtained from this study is in-line with the ones reported by [17] and [18]. The presence of genes encoding resistance to aminoglycosides, trimethoprim, sulphonamides, quinolones, tetracycline, macrolids, fosfomycin and β-lactams were reported in both fresh and archived clinical isolates except gene encoding resistance to rifampicin which was reported in only one of the fresh clinical isolate (Isolate 6 with ST 17). This is in agreement with the reports in some literatures where blaCTX-M type, blaSHV-type, blaTEM-type and FosA3 were the common genes implicated in the resistance of cephalosporins, monobactams, and fosfomycin identified in clinical and carriage *K. pneumoniae* in Lebanon and China [48]. This is an indication that ESB *K. pneumoniae* in either clinical or carriage could be probably resistance genes reservoir for other bacterial species and be responsible for genetic transfer to other species. The distribution or dissemination of ESBL-producing Kp in this healthcare setting could probably be attributed to a lack of prevention and control measures, effective infection for their containment [17].

One of the interesting findings of this study was the detection of 3789 unique gene codes and detection of CTX-M with only two variants (CTX-M-15_23 and CTX-M-55) and SHV-gene types with six variants (SHV-1_22, SHV-11_18, SHV-11, SHV-1_1.1, SHV-11_3 and SHV-11_19) as the most predominant beta-lactam genes which conform with the report that blaCTX-M as the predominant enzyme worldwide. In addition, resistance genes for quinolones (OqxA, OqxB, QnrB40-1, QnrS2, and QnrB10) were detected amongst the isolates. All the representative isolates of ESBL *K. pneumoniae* genomes showed high prevalence 6/6(100%) for OqxA and OqxB genes followed by QNrB40-1 with a prevalence of (50%) 4/6 then QnrS2 had (33.3%) 2/6 prevalence while only (16.7%) 1/6 prevalence was found in isolate 16 for QnrB10. These results obtained were an exception as they were higher than the report of 97% prevalence by [18] although high resistance to this particular class of antibiotic has been reported by other researchers in both Mexico and worldwide [49–51].

Regarding genes encoding resistance to tetracycline, the genomes of five ESBL *K. pneumoniae* representative showed high prevalence 5/6(83.3%) for tet(A)_4, this gene encoding resistance for tetracycline was not detected only in isolate 6. This finding was higher than the 45% prevalence reported by [18] and in disagreement in comparison with the lower ones reported in other studies [52]. In addition, fosfomycin resistance gene such as fosA3 was highly prevalent 5/6(83.3%) in almost all the representative isolates of ESBL-producing Kp genomes with exception of isolate 12 which had 16.7% prevalent each of the resistance genes floR2 and fosA7 fosfomycin resistance genes. This finding was in-line with the report of [17]. More so, resistance genes to aminoglycoside such as aac (3)-IIa, aac(6′)1b-cr-1, aac(6′)1b, aadA16, aph(3′)-VIa, aadA15, StrA4, StrB1 were identified in the genomes of all the representative strains of ESBL-producing Kp 6/6(100%). StrA4 and StrB1 recorded the highest prevalence 5/6(83.3%) each and were detected, this was followed by 2/6(33.3%) prevalent detection for aac(3)-IIa gene while 1/6(16.7%) each prevalent was recorded for aac(6′)1b, aadA16, aadA15and aph(3′)-VIa genes for 3 isolates. These findings were similar to the ones reported by [17, 18].

Trimethoprim resistance genes such as dfrA14 and dfrA27 were also detected. dfrA14 was the highest detected gene that conferred resistance to trimethoprim with 4/6(80%) prevalent and were detected in four of the representative strains of ESBL *K. pneumoniae* while 1/61(6.7%) prevalent of dfrA27 was detected in only one of the representative strains (6) of ESBL-producing Kp but no gene encoding resistance to trimethoprim was detected in representative stain (16) of ESBL-producing Kp. These findings also agreed with the reports of [17] and [18]. Furthermore, sulphonamide resistance encoding genes such as sul2_2, sul2_3 and sul1_11 were identified in isolates 3/6(1, 12 and 2), 1/6(B13) and 1/6(6) respectively, while no gene encoding resistance to sulphonamide was detected in isolate 0/6(16). The highest prevalence of 50% occurred in sul2_2 while 16.7% was prevalent for sul2_3 and sul1_11 each. This finding was not in agreement with sul1 and sul2 reported by [17]. However, this result is lower than 68% reported by [18].

Moreover, macrolid resistance gene such as mph(A)-1 was highly prevalent 3/6(50%) in the representative strains (16, 6 and B13) analysed in this study, while no gene encoding resistance to macrolid was detected in representative isolates 0/6(1, 2 and 12). In addition, the prevalence of gene encoding resistance to rifampicin was very low 1/6(16.7%) ARR-3 and only detected in one representative isolate (6) while not detected in five of the representative isolate 0/6(1, 2, 12, 16 and B13). The findings obtained from this research was in concurrent with the report of [17]. Meanwhile, all the representative strains presented at least one type of gene encoding for β-lactamases and the CTX-M and SHV-types were the most prevalent in sequenced genome (100%) each, two forms of CTX-M-type (blaCTX-M-15_23, blaCTX-M-55) were detected in the representative strains (1, 2, 6, 16 and B13), (12) with occurring prevalence of 83.3% and 16.7% respectively while 6 forms of SHV-type (blaSHV-1_22, blaSHV-11_18, blaSHV-11, blaSHV-1_1.1, blaSHV-11_3 and blaSHV-11_19) were detected in representative strains (1, 12, 16, 2, 6 and B13) respectively with 16.7% prevalent rate each. However, for

the TEM-type, highly prevalent (80%) of blaTEM-1_1 was detected in representative strains (1, 12, 2 and B13) while no TEM-type was detected in representative strains (16 and 6). The β-lactamases genes such as blaOXA-15_10, blaOXA-30_1 and blaNDM-1 were detected in representative strains (2, B13 and 6) respectively with 16.7% prevalence each. Another β-lactamases gene such as blaLEN6, blaLEN8 and blaLEN21 were detected in the genomes of representative strains (1, 12, 16), (16) and (B13) with prevalence of 50%, 16.7% and 16 > 7% respectively. Another interesting finding form this study was the detection of the β-lactamases gene (blaLEN) which was not detected or reported in many literatures and 80% prevalent of blaCTX-M-15 gene was detected in five representative strains genomes analyzed, which has been reported by different authors as common in nosocomial outbreaks [53–55]. All the representative strains (1, 12, 16, 2, 6 and B13) have at least one plasmid replicon (Table 4) and various virulence factors were detected from the representative *K. pneumoniae* genomes. Isolates 1 and 2 from sputum source, then 16 and 6 were from urine while isolate 12 was blood source but the source of B13 was out of the researcher's reach (Table 4).

The analysis of MLST profiles has shown high variation among the seven housekeeping genes and the most interesting finding from this study was detection of two novel sequence types (STs) of the representative strains (2 with ST NF* and 12 with ST NF) which their allele combinations were not found in the MLST database. However, four different sequence types (STs) of the representative strains were identified showing in general heterogeneous distribution which are: ST 394 (B13), ST 985 (1) ST11 (16) and ST17 (6) (Fig. 4). The two ESBL-producing Kp strains ST11 and ST17 (16 and 6) isolated from two patients respectively were detected in fresh clinical samples of urine, one ESBL-producing Kp strain ST985 (1) isolated from fresh clinical sample of sputum, the source of another ESBL-producing Kp ST394 (B13) isolated from a patient of archived clinical sample was urine, while two ESBL-producing Kp with novel sequence types (2, and 12) isolates from two patients were detected in both sputum and blood respectively of fresh clinical samples. The two novel sequence types (STs) representative strains (2 and 12) detected from the study also share most of the resistance genes detected. The result of MLST analysis obtained this study were entirely in disagreement with the sequence types (STs) reported by [17, 18] (Fig. 4).

All nucleotide sequences of the 34 isolates of *K. pneumoniae* were aligned in specific order and were subjected to phylogenetic analysis, among the 34 isolates, six were query isolates and five out of the six were labelled with identity numbers while one was labelled with letter followed by number but were all coloured red while 28 of the isolates were reference strains from NCBI database and were all labelled named with strain name (Fig. 5). The tree was basally rooted to strains (*K. pneumoniae* strain CMUH CRE003 and *K. pneumoniae* strain1, 2329), assigned as clade IV and branched out into three other clades I, II and III with 100% bootstrap of confidence interval values at all branching as shown in Fig. 4 above. Among the four clades, there was a major group consisting of 24 isolates from this study (clade I) while others were small clades comprising of 5, 3 and 2 clusters of isolates (clades II, III and IV) respectively. The clades also showed high level of close clonal relationship and evolutionary lineages. A clusters of close clonal relationship were mostly observed in clade II then clade III and finally in both clades IV and I. The ESBL-producing Kp isolates showed closed clonal relationship and lineages with other reference strains and were also distributed among clades but more importantly one of the isolates with novel sequence type (12) do not possessed clonal relationship with reference strains but share same lineage with some of the reference strains. These findings were in agreement with report of [56] and [17] but in contrary to the report of [18].

Conclusion

The results of this study indicated high prevalence of ESBL-producing *K. pneumoniae* in the study area as well as heterogeneous multi locus sequence types with multiple antibiotic resistance determinants. The diverse resistance genes revealed by this study is an alarming finding as these may increase the resistance rates over time unless stringent infection control measures and antibiotic stewardship programs are put in place or maintained to checkmate the spread of ESBL-producing *K. pneumoniae*.

Supplementary Information

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Supplementary Material 1
Supplementary Material 2
Supplementary Material 3
Supplementary Material 4
Supplementary Material 5
Supplementary Material 6
Supplementary Material 7

Author contributions

The conception of research idea (TZMTJ), Research work(SA), Research design (SA), Sequencing (SA, N M A/P S J V, SNM, MBA, and TZMTJ), Data analysis and interpretation of results (TZMTJ, SNM, HMN, MBA, NMA/PSJV, and SA), Manuscript drafting (SA), and review of the initial and final draft of the manuscript (TZMTJ, MNMD, HMN and SNM).

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Data availability
Sequence data that support the findings of this study have been deposited with GeneBank with accession code PRJNA1054040.

Declarations

Ethical approval
The ethical approval for the study was obtained from Medical Ethics Committee of the Universiti Putra Malaysia (Ref. No. UPM/TNCPI/RMC/JKEUPM/1.4.18.2 (JKEUPM) of the Universiti Putra Malaysia and Permission to conduct the research was also granted from the office of the director Hospital Sultan Abdul Aziz Shah (HSAAS) Universiti Putra Malaysia (UPM) (UPM/HPUPM/800-8).

Competing interests
The authors declare no competing interests.

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