Brazilian Journal of Microbiology (2020) 51:189–195
https://doi.org/10.1007/s42770-019-00208-w

CLINICAL MICROBIOLOGY - SHORT COMMUNICATION

Molecular characterization of extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* from a Malaysian hospital

Golnaz Mobasseri[1] · Kwai Lin Thong[1] · Ganeswarie Rajasekaram[2] · Cindy Shuan Ju Teh[3]

Received: 30 May 2019 / Accepted: 7 December 2019 / Published online: 14 December 2019

Abstract

Multidrug-resistant (MDR) and extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae* associated with nosocomial infections have caused serious problems in antibiotic management with limited therapeutic choices. This study aimed to determine the genotypic and phenotypic characteristics of *K. pneumoniae* strains isolated from a tertiary hospital in Malaysia. Ninety-seven clinical *K. pneumoniae* strains were analyzed for antimicrobial susceptibility, all of which were sensitive to amikacin and colistin (except one strain), while 31.9 % and 27.8 % were MDR and ESBL producers, respectively. PCR and DNA sequencing of the amplicons indicated that the majority of MDR strains (26/27) were positive for $bla_{TEM,}$ followed by bla_{SHV} (24/27), $bla_{CTX\text{-}M\text{-}1}$ group (23/27), $bla_{CTX\text{-}M\text{-}9}$ group (2/27), and *mcr-1* (1/27). Thirty-seven strains were hypervirulent and PCR detection of virulence genes showed 38.1 %, 22.7 %, and 16.5 % of the strains were positive for *K1*, *wabG*, and *uge* genes, respectively. Genotyping by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) showed that these strains were genetically diverse and heterogeneous. Sequence types, ST23, ST22, and ST412 were the predominant genotypes. This is the first report of colistin-resistant *K. pneumoniae* among clinical strains associated with *mcr-1* plasmid in Malaysia. The findings in this study have contributed to the effort in combating the increase in antimicrobial resistance by providing better understanding of genotypic characteristics and resistance mechanisms of the organisms.

Keywords ESBL · *Klebsiella pneumoniae* · Multidrug resistance · MLST · PFGE

Introduction

Extended-spectrum β-lactamases (ESBLs) are enzymes produced by Gram-negative bacteria to confer resistance to aminopenicillins, cephalosporins (first-, second-, third-, and fourth-generation), and aztreonam which are inhibited by clavulanic acid [1, 2]. Members of TEM, SHV, and CTX-M groups are the common families of β-lactamases which are found in *Escherichia coli*, *K. pneumoniae*, and other *Enterobacteriaceae* worldwide [1, 2]. These resistance determinants are usually plasmid-encoded. Among the ESBL enzymes, CTX-M type has been increasingly reported [1, 3]. In Malaysia, Palasubramaniam et al. (2005) reported an association of $bla_{SHV\text{-}5}$ ESBL gene in *K. pneumoniae* with a nosocomial outbreak, followed by several other reports [4–6].

Carbapenems have been used as the last-line antimicrobial drugs to treat serious infections caused by ESBL-producing *Enterobacteriaceae*; however, the emergence of carbapenemase-producing *Enterobacteriaceae* worldwide have left clinicians with very limited therapeutic antibiotic options [6, 7]. Therefore, early detection of ESBL- and carbapenemase-producing pathogens is the first step towards better infection control management [8]. ESBL-producing *K. pneumoniae* causing nosocomial infections such as pneumonia, urinary tract infections, septicemia, and soft tissue is a worldwide problem [1, 2]. The clonal complex 258

Responsible Editor: Mara Correa Lelles Nogueira.

✉ Cindy Shuan Ju Teh
cindysjteh@um.edu.my

[1] Institute of Biological Science, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia

[2] Department of Pathology, Hospital Sultanah Aminah Johor Bahru, 80100 Johor Bahru, Malaysia

[3] Department of Medical Microbiology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

(CC258), which is the predominant sequence type 258 (ST258), and single-locus variants of ST258 such as ST340, ST437, ST11, and ST512 have been reported to produce ESBLs [9].

Since the increase in carbapenemase-producing *K. pneumoniae* worldwide, colistin and tigecyclin have been used for treating infections caused by these resistant organisms [10, 11]. Unfortunately, the overuse of colistin has also resulted in increased colistin-resistant strain worldwide [10]. The main cause of resistance to colistin and tigecyclin is lipopolysaccharide (LPS) modification and is associated with mutations in *mgrB* and the two-component systems, *pho*PQ and *pmr*AB [12].

The plasmid-mediated colistin resistance due to *mcr-1* resistance gene was first reported by Liu et al. (2016) from food animals and patients recently in China which is followed by several reports in animals and humans worldwide [13–15].

In addition, several virulence factors have been detected in *K. pneumoniae* such as capsular serotypes, *magA*, *K2*, *rmpA* (the mucoid phenotype regulator) genes, *kfu* (responsible for an iron uptake system), *wabG* (responsible for biosynthesis of the outer core lipopolysaccharide), *uge* (responsible for biosynthesis of the capsule and smooth lipopolysaccharide), and *all*S which is associated with allantoin metabolism [16]. These virulence factors are associated with the ability to cause severe community-acquired infections such as liver abscesses, pneumonia, and meningitis in young healthy hosts and the ability to cause metastatic infections [16].

There is a need to have a more comprehensive data on the presence of ESBLs, virulence genes, and antimicrobial susceptibility trends of clinical *K. pneumoniae* strains in Malaysia to improve treatment options for a wide range of infections caused by this pathogen. Therefore, the objective of this study was to determine the antimicrobial susceptibility, resistance genes, virulence genes, and genetic diversity of clinical strains of *K. pneumoniae* isolated from a hospital in Johor Bahru, Malaysia.

Materials and methods

Ninety-seven non-repeat clinical *K. pneumoniae* strains previously isolated from patients (males, $n = 62$ and female, $n = 35$) admitted to the hospital from September to December 2014 were analyzed. These strains were archived laboratory cultures previously collected from the hospital. There is no personal information about patients. The only information are sources and gender. The strains were cultured from blood ($n = 25$), bronchoscopic aspirates (BBA) ($n = 24$), wound tissue ($n = 9$), swab sample ($n = 10$), urine ($n = 8$), pus ($n = 6$), poc ($n = 3$), sputum ($n = 8$), fluid ($n = 2$), slough ($n = 1$), and bone ($n = 1$). Identification of *K. pneumoniae* was performed by PCR using specific primers [17]. The species were confirmed by PCR targetting the *mdh* housekeeping gene (http://bigsdb.web.pasteur.fr/klebsiella/primers_used.html). All strains were cultured in Luria-Bertani broth and kept in 50% glycerol at − 20 °C. The PCR amplicons were purified and sequenced for validation of their identity.

The susceptibility of the *K. pneumoniae* strains to 16 antimicrobial agents, including cefoperazone (30 μg), ciprofloxacin (5 μg), ampicillin (10 μg), aztreonam (30 μg), piperacillin/tazobactam (10 μg), imipenem (10 μg), amikacin (30 μg), ceftazidime (30 μg), gentamycin (10 μg), colistin (10 μg), tetracycline (30 μg), cefotaxime (30 μg), sulbactam-cefoperazone (150 μg), amoxicillin-clavulanate (20/10 μg), meropenem (10 μg), and cefixime (5 μg) (Oxoid Limited Basingstoke, Hampshire, England) were determined by the disk diffusion method according to the Clinical and Laboratory Standard Institute (CLSI) guidelines [18].

ESBL production was confirmed using disk diffusion method as described in the CLSI guideline [18] and *E. coli* strain ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as quality control. Modified Hodge Test (MHT) was carried out to detect carbapenemase production in *K. pneumoniae* strains according to the CLSI guideline [19] and ATCC® BBA-1705 and ATCC® BBA-1706 were used as positive and negative controls, respectively. The minimum inhibitory concentration (MICs) for ceftazidime, amoxicillin-clavulanate, cefotaxime, meropenem, and imipenem (BioMerieux) was determined by E-test according to the CLSI guidelines and broth microdilution method (for colistin-resistant strain; KP2014C56) was performed for colistin. *E. coli* ATCC 25922 was used as a quality control strain [18].

Genomic DNA of all MDR *K. pneumoniae* strains were extracted by using DNA extraction kit (Yeastern Biotech Co., Ltd.) and subjected to PCR detection of β-lactamase genes including bla_{TEM}, bla_{SHV}, $bla_{\text{CTXM-1}}$ group, $bla_{\text{CTXM-2}}$ group, $bla_{\text{CTX-M-9}}$ group, $bla_{\text{OXA-1}}$, and $bla_{\text{OXA-9}}$; carbapenem resistance genes bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}, and $bla_{\text{OXA-48}}$; and colistin resistance genes *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, and *mcr-5* [13, 20, 21]. All PCR products were sequenced to validate their identity and the strains with the confirmed amplicons were used as positive controls for subsequent PCR analysis.

String test was performed for all 97 *K. pneumoniae* strains to distinguish hypervirulent *K. pneumoniae* (hvKP) from classical *K. pneumoniae* (cKP) [22]. Genomic DNA of all 97 *K. pneumoniae* strains were used for PCR detection of virulence genes including *K*1, *K*2, *uge*, *wabG*, *fimH*, *magA*, *rmpA*, and *kfu* [23]. Positive PCR-amplified products were sequenced to validate their identity and the strains with the correct amplicons were used as positive controls for subsequent PCR analysis.

Plasmid DNA from ESBL-producing *K. pneumoniae* strains were extracted using the alkaline lysis method which contains DNase (10 mg/mL) to avoid chromosomal DNA in the extracted plasmid. [24], followed by electrophoresis on a 1.5% agarose gel for 4.5 h at 80 V (3.2 V/cm). A 1 kb DNA ladder and lambda DNA/HindIII (Promega, Madison,WI USA) were used as DNA markers and plasmid extraction also was performed by using plasmid extraction kit (Qiagen), this experiment was repeated 3 times to confirm the results. The plasmid linearization and confirmation of the plasmid sizes were performed by S1 nuclease PFGE and lambda DNA; low-range DNA markers and *E. coli* V517 strain were used as standard reference [25]. PCR detection of selected β-lactamase genes (bla_{SHV}, bla_{TEM}, bla_{CTXM-1} group, and bla_{CTXM-9} group) and colistin resistance gene (*mcr-1*) was performed using extracted plasmid DNA. The PCR products were sequenced to confirm their identity [25, 26].

Transfer of ESBL and *mcr-1* genes by conjugation was performed in Luria-Bertani broth using nalidixic acid–resistant *E. coli* DH5α as the recipient strain and conjugation experiment was repeated 5 times to confirm the results. Transconjugants were selected on LB agar supplemented with nalidixic acid (100 mg/mL) and cefotaxime (2 mg/L) or colistin (1 mg/L) (Sigma Aldrich) [25, 26]. PCR detection of selected β-lactamase genes (bla_{SHV}, bla_{TEM}, bla_{CTXM-1} group, bla_{CTXM-9} group, and *mcr-1*) was performed using plasmid DNA extracted from transconjugants. The plasmid linearization was performed by S1 nuclease PFGE and lambda DNA; low-range DNA markers were used as the standard reference [25].

PFGE typing was carried out according to Lim et al. (2009) with minor modification [5]. In brief, equal volumes of the standardized cell suspensions ($OD_{610} = 0.6$) and 1% Seakem Gold agarose were mixed gently and allowed to solidify to form agarose plugs. The plugs were lysed with cell lysis buffer (50 mM Tris, 50 mM EDTA (pH = 8), 1% sarcosine, 1 mg/mL proteinase K (Promega, Madison, WI, USA)) and incubated for 4 h at 54 °C. The lysed plugs were then washed twice with sterile double distilled water and six times with TE buffer. The DNA agarose plugs were digested with 10 U of *Xba*I (Promega. Madison, WI USA) for 24 h at 37 °C and electrophoresed by using a CHEF-Mapper (BioRad, USA) with pulse times of 2.25–54.2 s at 6 V/cm for 24 h. Analysis of the PFGE banding patterns based on the unweighted pair group method was carried out using the BioNumerics 6.0 software; the DNA marker, *Salmonella* serotype Braenderup H9812 has been incorporated in the gel (first, middle, and last lanes) for linearization purpose with tolerance of 1.5.

Genotyping of 97 *K. pneumoniae* strains was determined by multilocus sequence typing (MLST) analysis. MLST was performed with seven housekeeping genes (*tonB*, *rpoB*, *pgi*, *phoE*, *infB*, *mdh*, and *gapA*). Alleles and sequence types were allocated by using the MLST database (http://bigsdb.web.pasteur.fr/perl/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_seqdef_public).

eBURST V3 (http://eburst.mlst.net) analysis using the most stringent definition, where the STs were identical or shared at least six alleles, was used to detect clonal complex or BURST groups (BGs) among the STs in this study and the *K. pneumoniae* MLST database. The STs were then categorized as BG founders, single-locus variants (SLVs), double-locus variants, and singletons [27].

Results and discussion

All 97 *K. pneumoniae* were susceptible to amikacin and colistin except for one strain (KP2014C56) that showed resistance to colistin. This strain, from a swab sample of an infected wound, was detected as MDR and ESBL-producing (multiple drug-resistant to aztreonam, sulbactam-cefoperazone, tetracycline, ampicillin, ceftazidime, cefotaxime, cefixime, and amoxicillin-clavulanate), hypervirulent, and harbored resistance genes $bla_{CTXM-15}$, bla_{TEM-1},bla_{SHV-11}, and *mcr-1*, and virulence genes *K1* and *wab*G. This strain had a unique pulstotype and belonged to ST65. In Malaysia, this is the first report of colistin-resistant *K. pneumoniae* among clinical strain associated with plasmidial *mcr-1*, which was recently reported in zoonotic *K. pneumoniae* from swine farms [20]. Polymyxin E (colistin) is effective against multidrug-resistant and carbapenemase-producing Gram-negative bacteria, but recently, colistin-resistant *Enterobacteriaceae* including *K. pneumoniae* has been reported worldwide [10, 12, 28]. The clinical *K. pneumoniae* strains from Greece (10.5–20%), Singapore (6.3%), South Korea (6.8%), and Canada (2.9%) showed highest resistance rate to colistin [10].

The percentages of antibiotic resistance for 97 *K. pneumoniae* strains are as follows: ampicillin (83.5%), cefotaxime (31.9%), tetracycline (30.9%), cefixime (29.8%), cefoperazone (27.8%), aztreonam (27.8%), ceftazidime (25.7%), amoxicillin-clavulanate (19.5%), tazobactam (11.3%), gentamycin (11.3%), sulbactam cefoperazone (8.2%), ciprofloxacin (7.2%), meropenem (6.1%), imipenem (2%), and colistin (1%). A majority of the *K. pneumoniae* strains (81/97) were resistant to ampicillin. There were 31 strains (31.9%) which showed resistance to more than three classes of antibiotics (qouinolone, monobactam, β-lactam, cephalosporin, polymyxin, aminoglycoside, tetracycline) and categorized as multidrug-resistant (MDR). Five strains showed carbapnem resistance based on AST and MIC results; KP2014C15 based on result of the disk diffusion method and MHT tests, 27 (27.8%) *K. pneumoniae* strains were ESBL producers and no carbapenemase-producing *K. pneumoniae* was detected.

These rates were higher than the rates reported in previous study which was carried out in 2000–2004 from another urban

general hospital (cefixime = 20.2%, cefotaxime = 18.1%, cefoperazone = 7.3%, and ceftazidime = 8%) [29]. However, it was lower than another study that was carried out in 2009 from five different hospitals in Peninsular Malaysia [5]. This could be due to different locations of the hospitals that serve different patient population. The current study only reported the resistance from a hospital in southern Malaysia while majority of the strains from previous studies were isolated from the central region.

The minimum inhibitory concentration (MIC) of cefotaxime and ceftazidime ranged from 16 to 256 μg/mL and from 8 to 256 μg/mL, respectively. Five strains of *K. pneumoniae* showed resistance to meropenem. All strains showed intermediate susceptibility and resistance to amoxicillin-clavulanate. Only one was resistant to colistin (16 μg/mL).

Multidrug-resistant and ESBL-producing *Enterobacteriaceae* of certain clonal complexes such as ST258 of *K. pneumoniae* have been disseminated worldwide [30]. The presence of virulence genes and drug resistance in these clonal strains of *K. pneumoniae* complicates treatment particularly among immunocompromised individuals [7, 31]. Previously, Low et al. (2017) reported the occurrence of carbapenem-resistant *K. pneumoniae* strains in another tertiary Malaysian hospital [6]. However, in this study, no carbapenemase-producing *K. pneumoniae* and no carbapenem resistance gene was detected.

Analysis of the DNA sequence of all the amplicons of ESBL-encoding genes in the 31 MDR *K. pneumoniae* strains showed that the majority (26/31) were positive for bla_{TEM}, all of which were identified as β-lactamase producer of TEM-1 enzyme. TEM-1 is a common β-lactamase among *Enterobacteriaceae* family [1]. This was followed by bla_{SHV} (24/31) which comprised of 9 bla_{SHV-11}, 7 bla_{SHV-12}, 6 bla_{SHV-28}, and 2 bla_{SHV-61}. Twenty-three MDR strains were $bla_{CTX-M-1}$ group (11 $bla_{CTX-M-15}$, 7 $bla_{CTX-M-1}$, 5 $bla_{CTX-M-28}$), two MDR had $bla_{CTX-M-9}$ and one MDR colistin-resistant strain had *mcr-1*. No amplification was observed for $bla_{CTX-M-2}$ group, bla_{OXA-1}, bla_{OXA-9}, bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}, and bla_{OXA-48} genes.

The CTX-M groups have been reported as predominant ESBL enzymes worldwide [1]. CTX-M-15 is one of the most common CTX-M-type ESBLs among the *Enterobacteriaceae* family. Nosocomial infections caused by CTX-M-15-producing *K. pneumoniae* have dramatically increased in recent years [1]. In Malaysia and other Asian countries, CTX-M-15 is the major ESBL enzyme reported [5, 32, 33].

According to antimicrobial susceptibility test and minimum inhibitory concentration results, five strains of *K. pneumoniae* showed resistance to meropenem ranged from 4 to 8 μg/mL (KP2014C15, KP2014C37, KP2014C62, KP2014C96, and KP2014C99) and only one strain (KP2014C96) showed resistance to imipenem (8 μg/mL).

Fig. 1 Cluster analysis of PFGE profiles for 97 *K. pneumoniae* strains used in this study. [a]Bronchoscopic aspirates (BBA). [b]Cefoperazone (CFP), aztreonam (ATM), amikacin (AMK), sulbactam cefoperazone (SCF), meropenem (MEM), cefixime (CFM), amoxicillin-clavulanate (AMC), ciprofloxacin (CIP), ampicillin (Amp), tazobactam (TZP), imipenem (IMP), ceftazidime (CAZ), colistin (CT), cefotaxime (CTX), gentamycin (GEN), and tetracycline (TET). [c]Female (F) or male (M)

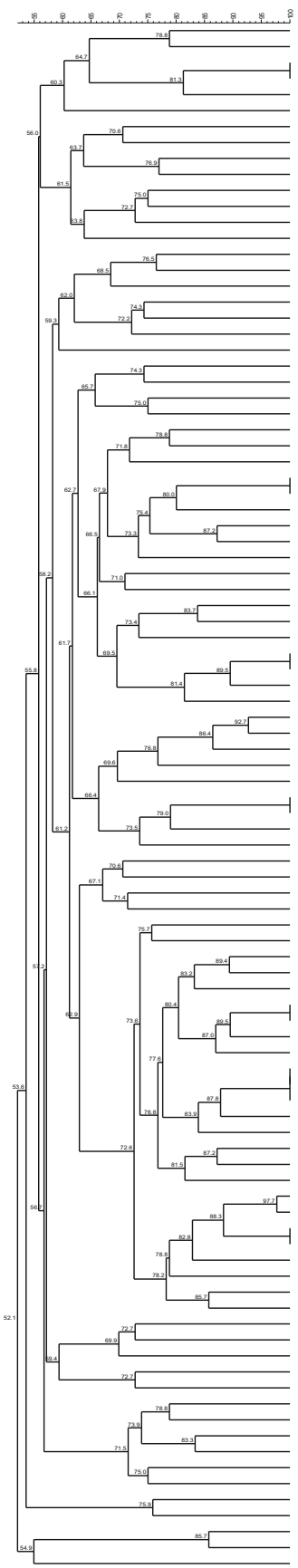
However, these 5 strains were MHT negative and carbapenemase genes were not detected. This finding suggested that the carbapenem resistance was due to different mechanisms and the possible mechanisms will be determined in future.

Thirty-seven strains (38.1%) were hypervirulent as they were positive for the string test, and all had the virulence gene, *K1*. Among 97 clinical strains, 22 (22.7%) and 16 (16.5%) were positive for *wabG* and *uge* genes, respectively. No *fimH*, *magA*, *rmpA*, *kfu*, and *K2* gene was detected.

Liu et al. (2018) reported the occurrence of virulence genes *K1* (34.4%), *K2* (20.8%), *rmpA1* (79.2%), *rmpA2* (70.8%), and *magA* (80.2%) among hvKP in China [13]. Lin et al. (2014) also reported the occurrence of *rmpA* (100%), *iuc* (96%), and *kfu* (11.5%) in *K. pneumoniae* strains from Singapore, Hong Kong, and Taiwan [33].

Plasmid analysis showed that all 27 ESBL-producing *K. pneumoniae* strains had plasmids with sizes ranging from 1500 to 20,000 bp; PCR detection of selected β-lactamase and colistin genes (bla_{SHV}, bla_{TEM}, bla_{OXA-1}, bla_{CTXM-1}, and *mcr-1*) using extracted plasmid DNA as templates showed 21 strains had bla_{TEM} in plasmids, 18 strains had bla_{SHV}, 15 strains had bla_{CTXM-1}, and one strain had *mcr-1* (KP2014C56), that this strain had 4 plasmids with sizes of 2500, 6000, 8000, and 20,000 bp that harbored $bla_{CTXM-15}$, bla_{TEM-1},bla_{SHV-11}, and *mcr-1*. Conjugation was carried out for all ESBL-producing and nalidixic acid–sensitive *K. pneumoniae* strains which harbored plasmids and ESBLs associated. Nineteen out of 27 were ESBL-encoding transconjugants. Plasmid analysis of the transconjugants confirmed that plasmids with sizes of 1500, 2500, 3000, 6000, 8000, 10,000, and 20,000 bp were transferred from the donors to the recipient, with each donor transferring between 2 and 4 plasmids to the recipient. Conjugation was carried out for colistin-resistant and nalidixic acid–sensitive *K. pneumoniae* strain (KP2014C56) which harbored plasmids, colistin, and ESBL genes. This strain was detected as colistin-resistant transconjugants. Plasmid analysis of the transconjugants confirmed that 3 plasmids with sizes of 2500, 8000, and 20,000 bp were transferred from the donors to the recipient which contained $bla_{CTXM-15}$, bla_{TEM-1},bla_{SHV-11}, and *mcr-1*.

Plasmid analysis and transconjugation experiments showed that all 27 ESBL-producing strains harbored plasmids and most of the ESBL genes were plasmid-encoded



Key	Source	[b]Antimicrobial resistance profile	[c]Sex	Resistance genes	Virulence genes
KP2014C52	[a]BBA	CFP,ATM,CAZ,CTX,CN,CFM,MEM,AMC	M	CTXM-15,CTXM-9,TEM-1,SHV-11	
KP2014C67	BBA	Amp	M		
KP2014C14	Blood	Amp	M		
KP2014C16	Blood	Amp	M		K1
KP2014C78	Peritaneal fluid	Amp	M		
KP2014C80	Poc	Amp	F		
KP2014C04	BBA	CFP,ATM,TE,CN,CAZ,CTX,CFM,AMC	M	CTXM-9,TEM-1,SHV-28	
KP2014C05	Blood	Amp	M		K1, uge
KP2014C21	Blood	TZP,TE	M		
KP2014C55	Swab	Amp	M		K1, wabG
KP2014C25	Blood	CAZ,CTX,TE,CFM,AMC	M		
KP2014C59	Tissue	Amp	F		
KP2014C01	Swab	Amp	M		
KP2014C99	Peritaneal fluid	CFP,ATM,Amp,CAZ,TE,CTX,CN,CFM,MEM,AMC	M	CTMX-15,TEM-1,SHV-11	K1, uge, wabG
KP2014C38	Sputum	Amp	M		K1
KP2014C92	Urine	Amp	F		
KP2014C18	Blood	CFP,ATM,CAZ,TE,CTX,CFM,AMC	M	CTXM-1,TEM-1,SHV-12	
KP2014C30	BBA	CFP,ATM,TZP,Amp,CAZ,TE,CTX,CFM,AMC	M	CTXM-15,TEM-1,SHV-11	
KP2014C48	BBA	Amp,TE	M		
KP2014C39	Poc	Amp	F		K1
KP2014C37	Sputum	CAZ,CTX,CFM,AMC,MEM	F	TEM-1,SHV-61	
KP2014C73	Blood	Amp	M		
KP2014C76	Slough	Amp	M		K1, wabG
KP2014C64	Pus	Amp	F		
KP2014C77	Blood	Amp	F		K1, wabG
KP2014C11	Blood	CTX,TE,CFM,AMC	F		
KP2014C28	Sputum	Amp	M		
KP2014C26	Blood	CFP,ATM,SCF,TZP,Amp,CAZ,TE,CTX,CFM,AMC	F	CTXM-1,TEM-1,SHV-28	K1
KP2014C46	Tissue	CFP,ATM,CIP,CAZ,TE,CTX,CFM,AMC	M	CTXM-15,TEM-1,SHV-11	K1, uge, wabG
KP2014C54	Swab	CFP,ATM,Amp,CAZ,CTX,TE,CFM,AMC	F	CTXM-1,TEM-1	
KP2014C53	Swab	Amp	M		uge, wabG
KP2014C13	Blood	CFP,ATM,CIP,TZP,CAZ.TE,CTX,CFM,AMC	F	CTXM-28,TEM-1,SHV-12	
KP2014C23	Pus	CFP,ATM,SCF,TZP,Amp,CAZ,TE,CTX,CFM,AMC	M	CTXM-15,TEM-1,SHV-11	uge
KP2014C100	Pus	Amp	M		
KP2014C51	BBA	Amp	M		K1
KP2014C57	Swab	Amp	F		
KP2014C10	Blood	CFP,ATM,CAZ,TE,CTX,CFM,AMC	M	TEM-1,SHV-28	K1, uge, wabG
KP2014C79	BBA	Amp	M		
KP2014C40	Tissue	CFP,ATM,CIP,SCF,TZP,Amp,CAZ,TE,CTX,IMP,CFM,.	F		K1, uge
KP2014C06	Urine	CFM,ATM,CIP,TE,CAZ,CTX,CFM,AMC	M	CTXM-15,TEM-1,SHV-12	
KP2014C47	Tissue	CFM,ATM,Amp,CAZ,TE,CTX,MEM,AMC	M	CTXM-1,TEM-1,SHV-11	
KP2014C12	Blood	Amp	F		
KP2014C56	Swab	ATM,SCF,CAZ,TE,CTX,CFM,AMC,CT	F	CTXM-1,TEM-1,SHV-11, MCR-1	K1, wabG
KP2014C17	BBA	Amp	M		
KP2014C88	Sputum	Amp	M		
KP2014C87	Pus	Amp	F		
KP2014C09	Blood	CFP,ATM,CIP,TZP,Amp,CN,CAZ,CTX,CFM,AMC	M	CTXM-15,TEM-1,SHV-12	K1, wabG
KP2014C71	Blood	CFP,Amp	F		K1, wabG
KP2014C15	Blood	CFP,ATM,CAZ,CTX,CFM,AMC,MEM	M	TEM-1,SHV-28	K1
KP2014C19	Bone	CFP,ATM,Amp,CAZ,CTX,CN,CFM,AMC	M	CTXM-1,TEM-1,SHV-12	K1, uge
KP2014C89	Urine	Amp,TE	M		wabG
KP2014C84	Urine	CFP,ATM,CIP,SCF,TZP,Amp,CAZ,TE,CTX,CN,CFM,.	F	CTXM-28,TEM-1,SHV-11	K1, wabG
KP2014C90	Blood	CFP,SCF,TZP,Amp,CAZ,TE,CTX,MEM,CFM,AMC	M	CTXM-15,TEM-1	K1, uge
KP2014C93	Urine	Amp,CIP	M		uge
KP2014C82	Swab	Amp	M		
KP2014C91	Swab	Amp,TE,CN,MEM,AMC	M		
KP2014C22	Blood	Amp	M		
KP2014C24	Blood	Amp	M		K1, uge, wabG
KP2014C33	BBA	Amp	F		
KP2014C83	Swab	CFP,ATM,CAZ,TE,CTX,CFM,MEM,AMC	F	CTXM-28,TEM-1,SHV-12	K1
KP2014C27	Sputum	Amp	M		K1, wabG
KP2014C03	Blood	Amp	M		
KP2014C49	BBA	Amp	F		K1, wabG
KP2014C63	BBA	Amp	F		K1, wabG
KP2014C43	Tissue	Amp	M		K1, uge
KP2014C29	BBA	Amp	M		wabG
KP2014C31	Blood	Amp	M		
KP2014C36	Urine	Amp	F		K1, uge, wabG
KP2014C02	Tissue	Amp	F		K1
KP2014C61	BBA	Amp	M		K1
KP2014C35	BBA	Amp	M		K1, uge
KP2014C95	BBA	Amp	M		
KP2014C34	BBA	Amp	M		
KP2014C08	Blood	Amp	F		
KP2014C72	Blood	Amp	M		
KP2014C68	BBA	CFP,ATM,Amp,CAZ,TE,CTX,CFM,MEM,AMC	M	CTXM-1,TEM-1,SHV-11	K1
KP2014C69	BBA	CFP,ATM,CAZ,TE,CTX,CFM,MEM,AMC	M	CTXM-28,TEM-1,SHV-28	
KP2014C58	Sputum	Amp	M		K1, uge, wabG
KP2014C74	Pus	CFP,ATM,Amp,CAZ,TE,CTX,CFM,MEM,AMC	F	CTXM-15,TEM-1,SHV-28	
KP2014C20	Blood	Amp	M		K1, wabG
KP2014C66	BBA	Amp	F		K1, uge, wabG
KP2014C07	Blood	Amp	M		
KP2014C85	Urine	Amp,TE	M		
KP2014C41	Tissue	Amp	M		K1
KP2014C32	BBA	CFM,ATM,TZP,CTX,CFM,AMC	M		
KP2014C70	Blood	Amp,TE	F		K1
KP2014C42	Tissue	Amp	F		
KP2014C96	BBA	CFP,ATM,SCF,Amp,CAZ,TE,CTX,CN,CFM,MEM,IPM,.	M	CTXM-15,TEM-1	
KP2014C86	Swap	Amp,MEM	F		
KP2014C94	Urine	ATM,CAZ,CTX,CFM,MEM,AMC	M	CTXM-28,SHV-12	
KP2014C62	BBA	CFP,ATM,SCFTZP,Amp,CAZ,TE,CTX.CN.MEM,AMC	F	CTXM-15,TEM-1,SHV-61	
KP2014C98	Sputum	Amp	M		
KP2014C44	Tissue	Amp	F		
KP2014C65	Pus	Amp	F		
KP2014C45	BBA	Amp	M		
KP2014C50	BBA	Amp	F		K1, wabG
KP2014C75	Poc	Amp	F		

and 19 strains had conjugative plasmids. These results concurred with previous studies on clinical *K. pneumoniae* which showed that ESBL genes carried on plasmids are transmissible [5, 34, 35]. Plasmid is one of the ways for the spread of ESBLs and other antibiotic resistance genes among microorganisms. Casper et al. (2017) reported the presence of ESBL genes and the *mcr-1* gene on a unique plasmid [36].

All the 97 strains were subtyped into 89 distinct pulsotypes comprising 14–27 restriction fragments. The genetic similarity of the strains ranged from 52.1 to 100%. The PFGE dendrogram showed 13 clusters (A to N) and 63 unique pulsotypes at 85% similarity cut off (Fig. 1). There was no direct association between pulsotypes with source of isolation (sampling source, kind of infection, and gender), virulence genes, and antimicrobial resistance phenotypes. This was not surprising as the strains were collected from different patients at different time points. Strains with high genetic similarity showed several antimicrobial susceptibility profiles. For instance, two strains in cluster B with identical pulsotypes but different antimicrobial resistance phenotypes. The 27 ESBL-producing *K. pneumoniae* strains yielded 23 unique pulsotypes. Generally, genotyping of 97 *K. pneumoniae* clinical strains showed high genetic diversity and heterogeneity.

MLST analysis of the *K. pneumoniae* strains yielded 24 different STs based on genetic variation in seven housekeeping genes. ST23 ($n = 20$) was a common ST among these strains. Other strains belonged to ST22 ($n = 7$), ST412 ($n = 7$), ST845 ($n = 6$), ST37 ($n = 5$), ST685($n = 5$) and ST336 ($n = 5$), ST1896 ($n = 4$), ST268 ($n = 4$), ST86 ($n = 4$), ST17 ($n = 3$), ST65 ($n = 3$), ST40 ($n = 3$), ST929 ($n = 3$), ST52 ($n = 3$), ST714 ($n = 2$), ST20 ($n = 2$), ST420 ($n = 2$), ST161 ($n = 2$), ST644 ($n = 2$), and ST29 ($n = 2$) and 3 unique STs such as ST426, ST592, and ST584. In comparison with the global *K. pneumoniae* MLST database by using eBURST V3, the Malaysian *K. pneumoniae* strains, ST22, ST23, ST17, ST20, and ST29, were international predicted founders. ST65 and ST37 were SLVs, while ST412, ST845, ST685, ST336, ST896, ST268, ST86, ST40, ST 929, ST52, ST714, ST420, ST161, ST644, ST426, ST592, and ST584 were singletons.

In conclusion, this study reports 31.9% MDR and 27.8% ESBL producers among 97 clinical *K. pneumoniae* strains. This is the first report of colistin resistance among clinical *K. pneumoniae* strains in Malaysia. No carbapenemase-producing *K. pneumoniae* strain was found, and bla_{SHV} and bla_{CTXM-1} were the predominant ESBL-encoding genes detected. PFGE typing showed diver subtypes circulating in the hospital. ST23, ST22, and ST412 and *wab*G, *uge*, and *K*1 were the predominant STs and virulence genes, respectively. These data indicated presence of diverse virulent MDR strains which might be useful for controlling and preventing the spread of antibiotic-resistant infections.

Acknowledgments We thank the University of Malaya for the financial support and facilities.

Funding information This work was supported by the University of Malaya Postgraduate Research Grant (PG072-2014B) and High Impact Research (HIR) Grant (UM.C/625/1/HIR/MOHE/CHAN/11/02).

Compliance with ethical standards

Conflict of interest The authors declare they have no conflict of interest.

References

1. Ali T, Ali I, Khan NA, Han B, Gao J (2018) The growing genetic and functional diversity of extended spectrum beta-lactamases. BioMed Res Int (Article ID 9519718). https://doi.org/10.1155/2018/9519718
2. Magiorakos AP, Srinivasan A, Carey R, Carmeli Y, Falagas M, Giske C, Harbarth S, Hindler J, Kahlmeter G, Olsson-Liljequist B (2012) Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect 18: 268–281
3. Iovleva A, Bonomo RA (2017) The ecology of extended-spectrum β-lactamases (ESBLs) in the developed world. J Travel Med 1;24(suppl_1):S44–S51. https://doi.org/10.1093/jtm/taw102
4. Palasubramaniam S, Subramaniam G, Muniandy S, Parasakthi N (2005) SHV-5 extended-spectrum beta-lactamase from *Klebsiella pneumoniae* associated with a nosocomial outbreak in a paediatric oncology unit in Malaysia. Int J Infect Dis 9:170–172
5. Lim KT, Yeo CC, Yasin RM, Balan G, Thong KL (2009) Characterization of multidrug-resistant and extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* strains from Malaysian hospitals. J Med Microbiol 58:1463–1469. https://doi.org/10.1099/jmm.0.011114-0
6. Low YM, Yap PS, Jabar KA, Ponnampalavanar S, Karunakaran R, Velayuthan R, Chong CW, Bakar SA, Yusof MY, Teh CS (2017) The emergence of carbapenem resistant *Klebsiella pneumoniae* in Malaysia: correlation between microbiological trends with host characteristics and clinical factors. Antimicrob Res Infect Cont 6(1):5. https://doi.org/10.1186/s13756-016-0164-x
7. Rodrigues C, Machado E, Ramos H, Peixe L, Novais Â (2014) Expansion of ESBL-producing *Klebsiella pneumoniae* in hospitalized patients: a successful story of international clones (ST15, ST147, ST336) and epidemic plasmids (IncR, IncFII K). Int J Med Microbiol 304:1100–1108. https://doi.org/10.1016/j.ijmm.2014.08.003
8. Hamzan NI, Yean CY, Rahman RA, Hasan H, Rahman ZA (2015) Detection of bla_{IMP4} and bla_{NDM1} harboring *Klebsiella pneumoniae* isolates in a university hospital in Malaysia. Emerg Health Threat J 1;8(1):26011. https://doi.org/10.3402/ehtj.v8.26011
9. Woodford N, Turton JF, Livermore DM (2011) Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. FEMS Microbiol Rev 35:736–755. https://doi.org/10.1111/j.1574-6976.2011.00268.x
10. Ah YM, Kim AJ, Lee JY (2014) Colistin resistance in *Klebsiella pneumoniae*. Int J Antimicrob Agents 1;44(1):8–15. https://doi.org/10.1016/j.ijantimicag.2014.02.016
11. Gupta N, Limbago BM, Patel JB, Kallen AJ (2011) Carbapenem-resistant *Enterobacteriaceae*: epidemiology and prevention. Clin Infect Dis 53(1):60–67. https://doi.org/10.1093/cid/cir202

12. Olaitan AO, Morand S, Rolain JM (2014) Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. Front Microbio 26(5):643
13. Liu C, Shi J, Guo J (2018) High prevalence of hypervirulent *Klebsiella pneumoniae* infection in the genetic background of elderly patients in two teaching hospitals in China. Infect Drug Res J 11:1031–1041. https://doi.org/10.2147/IDR.S161075
14. Olaitan AO, Diene SM, Kempf M, Berrazeg M, Bakour S, Gupta SK, Thongmalayvong B, Akkhavong K, Somphavong S, Paboriboune P (2014) Worldwide emergence of colistin resistance in *Klebsiella pneumoniae* from healthy humans and patients in Lao PDR, Thailand, Israel, Nigeria and France owing to inactivation of the *Pho*P/*Pho*Q regulator *mgr*B: an epidemiological and molecular study. Int J Antimicrob Agents 44:500–507
15. Hasman H, Hammerum AM, Hansen F, Hendriksen RS, Olesen B, Agersø Y, Zankari E, Leekitcharoenphon P, Stegger M, Kaas RS (2015) Detection of mcr-1 encoding plasmid-mediated colistin-resistant *Escherichia coli* isolates from human bloodstream infection and imported chicken meat, Denmark. Euro Surveill 20(49):261–262. https://doi.org/10.2807/1560-7917.ES.2015.20.49.30085
16. Yu WL, Ko WC, Cheng KC, Lee CC, Lai CC, Chuang YC (2008) Comparison of prevalence of virulence factors for *Klebsiella pneumoniae* liver abscesses between isolates with capsular *K1/K2* and non-*K1/K2* serotypes. Diagn Microbiol Infect Dis 62:1–6. https://doi.org/10.1016/j.diagmicrobio.2008.04.007
17. Diancourt L, Virginie P, Jan V, Patrick ADG, Sylvain B (2005) Multilocus sequence typing of Klebsiella pneumoniae nosocomial isolates. J Clin Microbiol 43(8):4178–4182
18. CLSI (2018) Performance standards for antimicrobial susceptibility testing; 28th informational supplement, document M100-S28. Clinical and Laboratory Standards Institute, Wayne
19. CLSI (2016) Performance standards for antimicrobial susceptibility testing; 26th informational supplement, document M100-S26. Clinical and Laboratory Standards Institute, Wayne Available from: http://www.facm.ucl.ac.be/intranet/CLSI/CLSI-2016-M100-S26.pdf
20. Mobasseri G, Teh CS, Ooi PT, Thong KL (2019) The emergence of colistin-resistant *Klebsiella pneumoniae* strains from swine in Malaysia. J Glob Antimicrob Resist, ISSN 2213-7165. https://doi.org/10.1016/j.jgar.2018.12.015
21. Rebelo AR, Bortolaia V, Kjeldgaard JS, Pedersen SK, Leekitcharoenphon P, Hansen IM, Guerra B, Malorny B, Borowiak M, Hammerl JA, Battisti A (2018) Multiplex PCR for detection of plasmid-mediated colistin resistance determinants, *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5* for surveillance purposes. Euro Surveill 23(6):17–00672
22. Qu TT, Zhou JC, Jiang Y, Shi KR, Li B, Shen P, Wei ZQ, Yu YS (2015) Clinical and microbiological characteristics of *Klebsiella pneumoniae* liver abscess in East China. BMC Infect Dis 15(1):161. https://doi.org/10.1186/s12879-015-0899-7
23. Qiong C, Zou JW, Qiu CN, Wang MM, Wang XJ, Ruan Z, Fan JZ (2018) Antimicrobial susceptibility and microbiological and epidemiological characteristics of hypermucoviscous *Klebsiella pneumoniae* strains in a tertiary hospital in Hangzhou, China. J Glob Antimicrob Resist 15:61–64
24. Feliciello I, Chinali G (1993) A modified alkaline lysis method for the preparation of highly purified plasmid DNA from *Escherichia coli*. Anal Biochem 212:394–401
25. Lai K, Ma Y, Guo L, An J, Ye L, Yang J (2017) Molecular characterization of clinical IMP-producing *Klebsiella pneumoniae* isolates from a Chinese tertiary hospital. Ann Clin Microbiol Antimicrob 16(1):42
26. Anjum MF, Duggett NA, AbuOun M, Randall L, Nunez-Garcia J, Ellis RJ, Rogers J, Horton R, Brena C, Williamson S (2016) Colistin resistance in *Salmonella* and *Escherichia coli* isolates from a pig farm in Great Britain. J Antimicrob Chemother 71(8):2306–2313. https://doi.org/10.1093/jac/dkw149
27. Spratt BG, Hanage WP, Li B, Aanensen DM, Feil EJ (2004) Displaying the relatedness among isolates of bacterial species–the eBURST approach. FEMS Microbiol Lett 1:241(2):129–134
28. Capone A, Giannella M, Fortini D, Giordano A, Meledandri M, Ballardini M, Venditti M, Bordi E, Capozzi D, Balice MP (2013) High rate of colistin resistance among patients with carbapenem-resistant *Klebsiella pneumoniae* infection accounts for an excess of mortality. Clin Microbiol Infec 19:E23–E30. https://doi.org/10.1111/1469-0691.12070
29. Loh LC, Chin H, Chong Y, Jeyaratnam A, Raman S, Vijayasingham P, Thayaparan T, Kumar S (2007) *Klebsiella pneumoniae* respiratory isolates from 2000 to 2004 in a Malaysian hospital: characteristics and relation to hospital antibiotics consumption. Singap Med J 48(9):813–818
30. Bonnedahl J, Hernandez J, Stedt J, Waldenström J, Olsen B, Drobni M (2014) Extended-spectrum β-lactamases in *Escherichia coli* and *Klebsiella pneumoniae* in gulls, Alaska, USA. Emerg Infect Dis 20:897–899. https://doi.org/10.3201/eid2005.130325
31. Ramos PIP, Picão RC, de Almeida LGP, Lima NCB, Girardello R, Vivan ACP, Xavier DE, Barcellos FG, Pelisson M, Vespero EC (2014) Comparative analysis of the complete genome of KPC-2-producing *Klebsiella pneumoniae* Kp13 reveals remarkable genome plasticity and a wide repertoire of virulence and resistance mechanisms. BMC Genomics 15:1–16. https://doi.org/10.1186/1471-2164-15-54
32. Lee MY, Kwan SK, Cheol-In K, Doo RC, Kyong RP, Jae HS (2011) High prevalence of CTX-M-15-producing *Klebsiella pneumoniae* isolates in Asian countries: diverse clones and clonal dissemination. Int J Antimicrob Agents 38:160–163. https://doi.org/10.1016/j.ijantimicag.2011.03.020
33. Mohd Helmi U, Desa M, Taib N, Tengku Jamaluddin T, Masri S (2016) Multiple ambler class A ESBL genes among *Klebsiella pneumoniae* isolates in a Malaysian district hospital. Trop Biomed 33(1):109–119
34. Göttig S, Gruber TM, Stecher B, Wichelhaus TA, Kempf VAJ (2015) In vivo horizontal gene transfer of the carbapenemase OXA-48 during a nosocomial outbreak. Clin Infect Dis 60(12):1808–1815. https://doi.org/10.1093/cid/civ191
35. Ho WS, Yap KP, Yeo CC, Rajasekaram G, Thong KL (2016) The complete sequence and comparative analysis of a multidrug-resistance and virulence multireplicon IncFII plasmid pEC302/04 from an extraintestinal pathogenic Escherichia coli EC302/04 indicate extensive diversity of IncFII plasmids. Front Microbiol 11(6):1547
36. Caspar Y, Maillet M, Pavese P, Francony G, Brion JP, Mallaret M-R, Bonnet R, Robin F, Beyrouthy R, Maurin M (2017) Mcr-1 colistin resistance in ESBL-producing *Klebsiella pneumoniae*, France. Emerg Infect Dis 23(5):874–876