



First genomic insights into carbapenem-resistant *Klebsiella pneumoniae* from Malaysia

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ABSTRACT

Objectives: Despite the increasing reports of carbapenem-resistant Enterobacteriaceae in Malaysia, genomic resources for carbapenem-resistant clinical strains of *Klebsiella pneumoniae* (*K. pneumoniae*) remain unavailable. This study aimed to sequence the genomes of multiple carbapenem-resistant *K. pneumoniae* strains from Malaysia and to identify the genetic basis for their resistance.

Methods: Illumina whole genome sequencing was performed on eight carbapenem-resistant *K. pneumoniae* isolated from a Malaysian hospital. Genetic diversity was inferred from the assembled genomes based on in silico multilocus sequence typing (MLST). In addition, plasmid-derived and chromosome-derived contigs were predicted using the machine learning approach. After genome annotation, genes associated with carbapenem resistance were identified based on similarity searched against the ResFinder database.

Results: The eight *K. pneumoniae* isolates were grouped into six different sequence types, some of which were represented by a single isolate in the MLST database. Genomic potential for carbapenem-resistance was attributed to the presence of plasmid-localised *bla*_{NDM} (*bla*_{NDM-1}/*bla*_{NDM-5}) or *bla*_{KPC} (*bla*_{KPC-2}/*bla*_{KPC-6}) in these sequenced strains. The majority of these carbapenem resistance genes was flanked by repetitive (transposase or integrase) sequences, suggesting their potential mobility. This study also reported the first *bla*_{KPC-6}-harbouring plasmid contig to be assembled for *K. pneumoniae*, and the second for the genus *Klebsiella*.

Conclusion: This study reported the first genomic resources for carbapenem-resistant *K. pneumoniae* from Malaysia. The high diversity of carbapenem resistance genes and sequence types uncovered from eight isolates from the same hospital is worrying and indicates an urgent need to improve the genomic surveillance of clinical *K. pneumoniae* in Malaysia.

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1. Introduction

Carbapenems remain the first-line therapeutic antimicrobials for severe infections caused by organisms such as extended-spectrum β -lactamase (ESBL)-producing multidrug-resistant Enterobacteriaceae

[1]. Therefore, the increasing worldwide trend of carbapenem-resistant Enterobacteriaceae (CRE) represents a formidable threat to modern healthcare and is associated with high morbidity and mortality [2]. In addition to the resistance to β -lactams, CREs are frequently resistant to most other classes of antibiotics, diminishing and even eliminating the efficacy of the available antibiotic armamentarium [1]. Amongst the risk factors associated with carbapenem resistance are previous use of carbapenems, underlying comorbidities, longer hospital stay, mechanical ventilation, intensive care unit (ICU) stay, surgeries, and transfer from healthcare settings with high rates of carbapenem resistance [1,2].

Among Enterobacteriaceae, *Klebsiella* spp. have emerged as the most important pathogens causing a wide range of healthcare-

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related infections, including pneumonia, bacteraemia, urinary tract and wound infections [3]. Over the last decades, carbapenem resistance has been steadily escalating among the Enterobacteriaceae, especially amongst the *Klebsiella* spp. [1]. Multiple carbapenem-resistance mechanisms have been identified, including the production of carbapenemases with direct carbapenem-hydrolysing activity, over-expression of efflux pumps, and reduced permeability of the outer membrane mediated by porin mutations. Three types of carbapenemases (class A: bla_{KPC}, bla_{GES}; class B: bla_{NDM-1}, bla_{IMP} and bla_{VIM}; and class D: bla_{OXA-48}) hydrolyse carbapenems at varying degrees [3]. Carbapenem genes are mainly carried on plasmids and pose a potential for widespread dissemination of carbapenem resistance, which describes their inclination to cause outbreaks of infection within and between healthcare facilities [2,4]. Understanding the molecular epidemiology and genetic characteristics of carbapenem-resistant *Klebsiella* strains from hospital environments requires a discriminatory bacterial typing technique. However, widely used methods such as pulsed-field gel electrophoresis or multilocus sequence typing (MLST) lack this discriminatory resolution [4].

Whole genome sequencing (WGS) elicits a level of discrimination on genetic relatedness that readily surpasses the previous typing methods [4]. However, there is a scarcity of reports on WGS of antibiotic-resistant clinical Enterobacteriaceae isolated from Malaysia and most have been mainly confined to genome sequencing of single isolates without in-depth comparative analysis [5–8]. The current study performed WGS of the eight carbapenem-resistant clinical *Klebsiella pneumoniae* (*K. pneumoniae*) strains isolated from a hospital in Malaysia, and provided the first insight into the genetic diversity and antimicrobial resistance mechanisms of carbapenem-resistant *K. pneumoniae* in Malaysia.

This data were especially important as resistance to imipenem and meropenem is on the rise in Malaysia: from 2.3% and 2.6%, respectively, in 2016 to 4.2% and 4.7%, respectively, in 2017 [9]. Whole genome sequencing provides important information to infer the origin and spread of *K. pneumoniae* strains and their antibiotic resistance genes in the healthcare setting, facilitating future epidemiological surveillance and infection control efforts in Malaysia.

2. Materials and Methods

2.1. Bacterial isolates and determination of MIC

Eight non-clonal clinical *K. pneumoniae* strains isolated from a hospital in Johor, Malaysia, were revived from -20°C glycerol stock cultures and grown on nutrient agar plates at 30°C for 48 h. These isolates were anonymised prior to DNA extraction and sequencing, and no clinical or demographic data were collected. These strains were sub-cultured onto the same medium and tested for an expanded range of antimicrobial sensitivities using the VITEK 2 system (BioMérieux, Marcy l'Etoile, France). Specifically, the VITEK 2 AST-GN87 cards were used to test for the following β -lactam antibiotics (concentration range): ampicillin/sulbactam (2/1–32/16 $\mu\text{g}/\text{mL}$), cefazolin (4–64 $\mu\text{g}/\text{mL}$), ceftriaxone (1–64 $\mu\text{g}/\text{mL}$), cefepime (1–64 $\mu\text{g}/\text{mL}$), ceftazidime (1–64 $\mu\text{g}/\text{mL}$), piperacillin/tazobactam (4/4–128/4 $\mu\text{g}/\text{mL}$), imipenem (0.25–16 $\mu\text{g}/\text{mL}$), meropenem (0.25–16 $\mu\text{g}/\text{mL}$), and ertapenem (0.5–8 $\mu\text{g}/\text{mL}$). The susceptibility category was designated according to the 2017 Clinical and Laboratory Standards Institute (CLSI) M100-S27 guidelines. *Escherichia coli* ATCC 25922 was used as the quality control strain for antibiotic susceptibility testing.

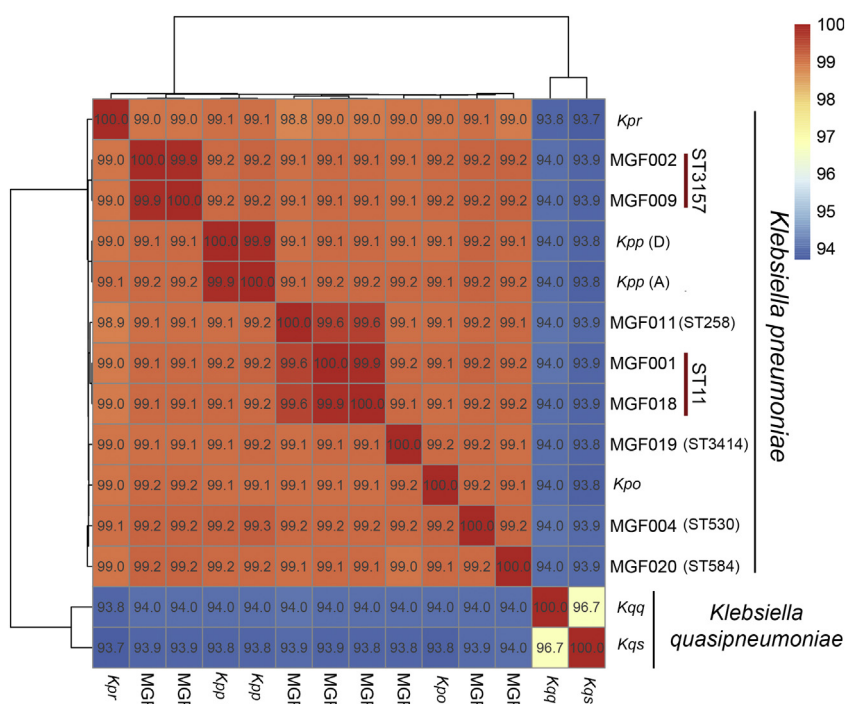


Fig. 1. A heatmap showing the hierarchical clustering of *Klebsiella pneumoniae* and *Klebsiella quasipneumoniae* strains based on genomic distance.

The sequence types of each isolate reported in this study are indicated either in brackets or next to the red vertical lines.

Values in boxes indicate pairwise average nucleotide identity (See Supplemental Table 1 for NCBI accession codes).

Abbreviations: Kpr, *K. pneumoniae* subsp. rhinoscleromatis ATCC 13884^T; Kpp(D), *K. pneumoniae* subsp. *pneumoniae* DSM 30104^T; Kpp(A), *K. pneumoniae* subsp. *pneumoniae* ATCC 13883^T; Kpo, *K. pneumoniae* subsp. *ozaene* ATCC 11296^T; Kqs, *K. quasipneumoniae* subsp. *similipneumoniae* 07A044^T; Kqq, *K. quasipneumoniae* subsp. *quasipneumoniae* 01A030^T.

Table 1
Characteristics and susceptibility profiles of carbapenemase-producing isolates.

Isolate	Sequence type	Isolation source	Isolation date (mm/dd/yy)	β-lactamase			Beta-lactam			2nd-generation cephalosporin		3rd-generation cephalosporin		4th-generation cephalosporin		Carbapenem		
				NDM	KPC	CTX-M	OXA	SHV	TEM	Ampicillin/sulbactam	Piperacillin/tazobactam	CFZ	CAZ	CTX	CFM	ETP	IMP	MEM
MGF001	11	Urine	03/15/12	1	15	15				≥32	≥128	≥64	≥64	≥64	≥64	≥8	8	4
MGF002	3157	Urine	04/10/12	5	15	15		187*	1C	≥32	≥128	≥64	≥64	≥64	≥64	≥8	≥16	≥16
MGF004	530	Urine	05/02/12	1	15	15		187*		≥32	≥128	≥64	≥64	≥64	≥64	≥8	≥16	≥16
MGF009	3157	Urine	04/07/15	5	15	15		187*	1C	≥32	≥128	≥64	≥64	≥64	≥64	≥8	≥16	≥16
MGF011	258	Body fluid	03/05/15		2		9*	182*		≥32	≥128	≥64	≥64	≥64	≥64	≥8	8	≥16
MGF018	11	Urine	08/18/16	1	15	15		187*	1B	≥32	≥128	≥64	≥64	≥64	≥64	≥8	≥16	8
MGF019	3414	Urine	08/15/16	1			9*			≥32	≥128	≥64	≥64	≥64	≥64	≥8	≥16	≥16
MGF020	584	Body fluid	09/13/16		6	15			1C	≥32	≥128	≥64	≥64	≥64	≥64	≥8	≥16	≥16

* <98% nucleotide identity to the reference gene.

2.2. Whole genome sequencing

Five to ten bacterial colonies were scraped from the 2-day-old agar plate culture, with a sterile P200 pipette tip, and used for genomic DNA extraction using a conventional SDS-based extraction method. The purified gDNA was quantified with Qubit 2 (Invitrogen, Santa Clara, CA), normalised to 0.2 ng/μL and processed using the Nextera XT library preparation kit (Illumina, San Diego, CA). Sequencing was performed on the Miseq (run configuration of 2 × 250 bp) located at the Monash University Malaysia Genomics Facility.

2.3. Genome assembly and analysis

Raw paired-end reads were adapter-trimmed using Trimmomatic v0.36 [10]. Error correction followed by de novo assembly of the trimmed reads used Unicycler v0.3.0 [11]. Genome-based species verification was performed with Jspecies v1.2.1 using the ‘mummer’ algorithm [12]. The average nucleotide identity matrix produced by Jspecies v1.2.1 was subsequently inputted into R to generate a clustered heatmap with the pheatmap package (default setting) [13,14]. Upon species confirmation, in silico multilocus sequence typing was performed on the assembled genomes using the software mlst v2.16 (<https://github.com/tseemann/mlst>) that scans the assembled contigs against the *K. pneumoniae* PubMLST typing schemes [15]. Screening of contigs for antimicrobial resistance genes against the Resfinder database (accessed 28 July 2018) used ABRicate v0.8.11 (<https://github.com/tseemann/abricate>) [16].

2.4. Identification and visualisation of plasmid-derived contigs

Mplasmids was used to accurately predict plasmid-derived and chromosomal-derived sequences in the genome assemblies based on pentamer frequency and the machine learning approach [17]. More specifically, mplasmids used the support-vector machine models that have been trained on various complete *K. pneumoniae* genomes to classify the origin of contigs, enabling accurate in silico localisation of antibiotic resistance genes to the plasmid or chromosome of *K. pneumoniae* isolates. Visualisation and alignment of the plasmid-derived contigs used BRIG v0.95 and EasyFig v2.1 [18,19].

3. Results

3.1. Genome assembly and statistics

The assembled genome size ranged from 5.23 to 5.64 Mb (GC content of 57.14–57.57%) (See Supplemental Table 1 for NCBI accession codes). Each strain exhibited >98% pairwise average nucleotide identity (ANI) to the currently described type strains of *K. pneumoniae* subspecies with consistently <95% pairwise ANI to *Klebsiella quasipneumoniae* subsp. *similipneumoniae* 07A044^T and *K. quasipneumoniae* subsp. *quasipneumoniae* 01A030^T [20] (Fig. 1). Within the ANI heatmap, minor clustering could be observed for isolates that exhibited strikingly high pairwise ANI (>99.8%) (e.g. MGF001 and MGF018, and MGF002 and MGF009). Although a relatively high pairwise ANI (>99%) was observed among *K. pneumoniae* subsp. *pneumoniae* DSM 30104^T, *K. pneumoniae* subsp. *ozaenae* DSM 16358^T and *K. pneumoniae* subsp. *rhinoscleromatis* DSM 16231^T, this was not the case for *K. quasipneumoniae* subsp. *similipneumoniae* 07A044^T and *K. quasipneumoniae* subsp. *quasipneumoniae* 01A030^T (pairwise ANI <96%) (Fig. 1).

3.2. Multiple genetic origins of *K. pneumoniae* isolates as revealed by in silico MLST

All *K. pneumoniae* MLST housekeeping genes (*rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB* and *tonB*) were complete and presented as a single

copy in the assembled *K. pneumoniae* genomes [21]. Although no novel sequence type was uncovered, which is somewhat unusual given the paucity of Malaysian *K. pneumoniae* strain representation in the MLST database, a high diversity of sequence types among the eight carbapenem-resistant strains was found. Five sequence types (ST) were identified: ST11 (MGF001 and MGF018), ST258 (MGF011), ST530 (MGF004), ST584 (MGF020), and ST3157 (MGF002 and MGF009), and strains forming minor clusters in the ANI heatmap were classified to the same ST consistent with their high genomic relatedness (Table 1). It is also worth noting that strains sharing the same ST also shared the same isolation source. For example, all strains from ST11, ST530, ST3157 and ST3414 were isolated from urine samples, while strains belonging to ST258 and ST584 were isolated from body fluid samples.

3.2.1. Evidence of genomic potential for carbapenemase production in the sequenced isolates

VITEKS2 assessment indicated that all isolates were highly resistant to the three tested carbapenems, namely ertapenem, imipenem and meropenem (Table 1). In addition to carbapenems, the isolates were also highly resistant to cephalosporins (cefazolin, ceftazidime, ceftriaxone, cefepime) and β -lactam/ β -lactamase inhibitor combinations (ampicillin-sulbactam, piperacillin-tazobactam). Genome mining for antibiotic resistance genes revealed that each strain harboured one of the two main classes of carbapenem resistance gene, namely *bla*_{NDM} and *bla*_{KPC} in addition to some ESBL genes (Table 1). Two known genetic variants were found for each of the carbapenemase genes. Based on current sampling, the *bla*_{NDM-1} gene was the most commonly found followed by *bla*_{NDM-5}. The distribution of *bla*_{NDM} and *bla*_{KPC} variants was not random and appeared to be ST-specific. It is also worth noting that, despite sharing the identical *bla*_{NDM-1} gene sequence with MGF004 and MGF019 belonging to ST530 and ST3414, respectively, MGF001 and MGF018 (both assigned to ST11) exhibited a substantially lower resistance (<16 μ g/mL) to the carbapenem drug, meropenem (Table 1).

3.2.2. Gene synteny in the neighbourhood of carbapenemase genes

Plasmid-derived contigs were identified in all eight strains, with cumulative lengths ranging from 226–580 kb (Supplemental Table 2). The contigs containing *bla*_{NDM} or *bla*_{KPC} were all classified as 'plasmid' by mlplasmids. Five of eight of the *bla*_{NDM}/*bla*_{KPC}-containing contigs were >15 kb, with the longest one being the *bla*_{KPC-6}-containing contig (83 kb) from strain MGF020 (Figs. 2 and 3). The three remaining plasmid contigs were <3200 bp in length and consisted of a *bla*_{NDM} gene and three upstream genes (*nagA*, *trpF* and *ble*) (Fig. 2). The gene cluster *nagA-trpF-ble* that encodes for alpha-N-acetylgalactosaminidase, anthranilate isomerase and bleomycin-binding protein was consistently found upstream of *bla*_{NDM} in the longer *bla*_{NDM}-containing contigs. In two of the *bla*_{NDM}-containing contigs (e.g. contig36_{MGF004} and contig37_{MGF019}) two genes both coding for integrase were located downstream of *bla*_{NDM} and arranged in tandem. On the contrary, *bla*_{KPC} was immediately flanked by transposase-coding genes transcribed in opposite orientation.

3.2.3. The first *K. pneumoniae* plasmid-derived contig harbouring the *bla*_{KPC-6} variant

Using the entire contig25 from the KPC-6-producing MGF020 isolate as the query to search against the NCBI non-redundant nucleotide database (NCBI nt database, accessed 27 February 2019), significant matches with high query coverage (>90% of query length) were found for a few *bla*_{KPC-2}-containing complete InA/C plasmid sequences. Interestingly, BLASTN search using only the annotated *bla*_{KPC-6} gene in contig25_{MGF020} as the query against the NCBI non-redundant nucleotide database (accessed 27 February 2019) returned only the reference CDS of *bla*_{KPC-6} (GenBank Accession Number: EU555534.1) with exact match (100% identity) followed by several 99.887% identity hits representing a single mismatch to the 882 bp *bla*_{KPC-6} gene to some *bla*_{KPC-2}-containing plasmids. Within the Enterobacteriaceae whole genome shotgun sequence database, an exact match of the *bla*_{KPC-6} gene was found to two contigs, JRTV01000008 and JRTV01000009, both from the genome assembly of *K. variicola* strain 223/14 also isolated from Malaysia.

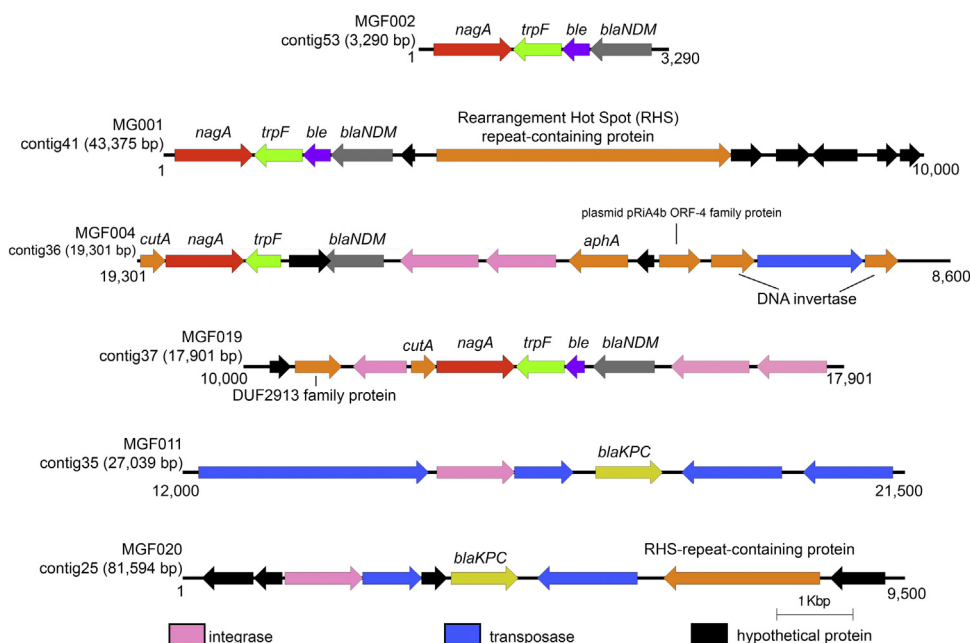


Fig. 2. The gene neighbourhood of carbapenemase genes.

Arrows of similar colour represent genes predicted to have similar functions. Direction of arrows indicates transcription orientation, and scale bar represents contig length.

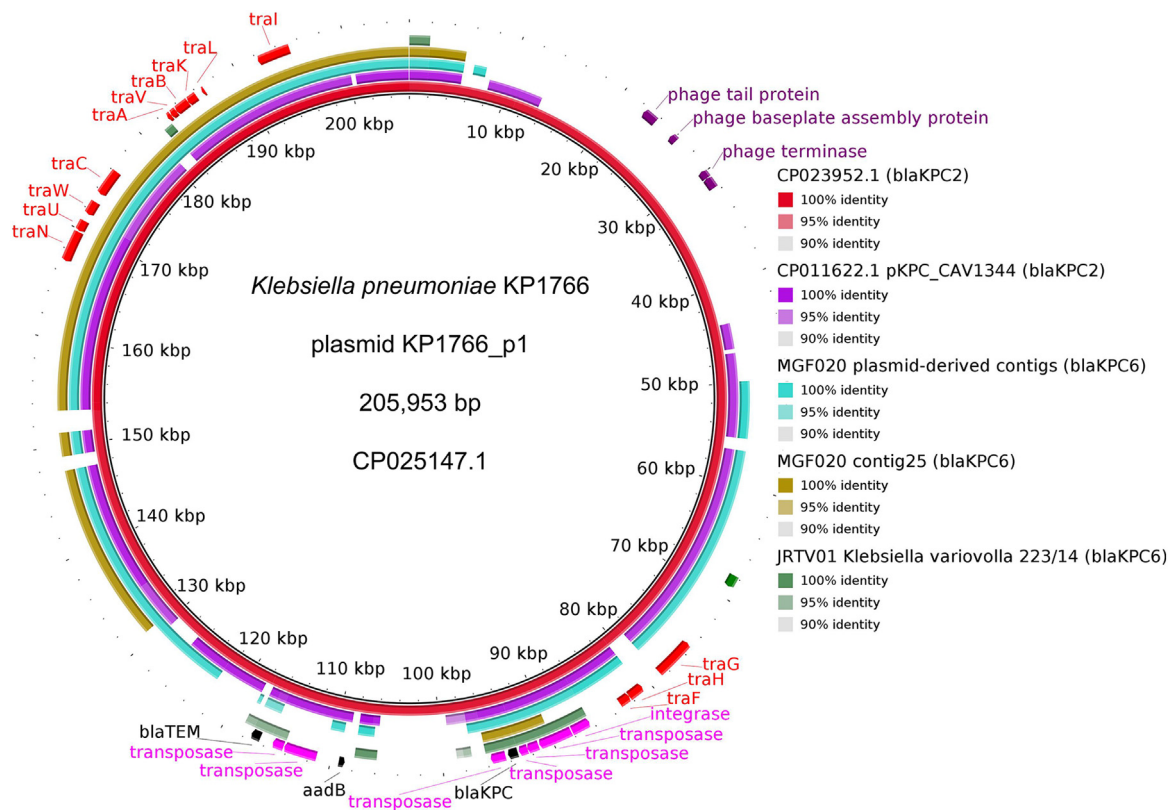


Fig. 3. Circular comparison of *bla*_{KPC} plasmids.

Each plasmid is represented by a coloured ring shaded based on nucleotide similarity to the reference plasmid KP1766_p1 (min. 90%; max. 100%).

The outermost ring highlights the gene regions involved in plasmid conjugation (*tra*), antibiotic resistance (*bla*_{TEM} and *bla*_{KPC}), phage assembly (red arrows), and gene mobility (pink arrows).

Using the plasmid-derived contigs from MGF020 as the query (Supplemental Table 2), a significant nucleotide alignment was observed (>90% nucleotide identity) across the entire plasmid KP1766_p1 of *K. pneumoniae* strain KP1766 (GenBank Accession: CP025147.1) except for the region ranging from 15 to 40 kb that consisted of phage-protein coding genes (purple outer arrows in Fig. 3). This region was similarly absent from the complete pKPC_CAV1344 plasmid of *K. pneumoniae* strain CAV1344 (GenBank Accession: CP011622.1). Contig25MGF020 covered approximately 30% length fraction of plasmid KP1766_p1 and contained the typical plasmid conjugation *tra* genes (red outer arrows in Fig. 3). Interestingly, despite harbouring the *bla*_{KPC-6} gene, very little significant sequence match to the reference IncA/C plasmid was observed when the whole genome assembly of Malaysian *bla*_{KPC6}-producing *K. variicola* was used as the query (Fig. 3).

4. Discussion

The genomes of eight *K. pneumoniae* isolates from a Malaysian hospital were assembled. The genomes represented a 100% increase in the number of publicly available Malaysian *K. pneumoniae* genomes (NCBI Assembly database as of 27 February 2019), underscoring the paucity of genomic representation of this clinically important bacterial species from Malaysia. In addition, these genomes were the first carbapenem-resistant *K. pneumoniae* genomes reported from Malaysia, although it is worth mentioning that the genomes of carbapenem-resistant *K. variicola* and *K. quasipneumoniae* isolated from Malaysia have also recently been published [5,6]. The high genetic diversity of *K. pneumoniae* in this current genomic sample, as indicated by the number of different

STs recovered from this sample, was unexpected given their identical sampling site, suggesting multiple introductions/origins of carbapenem-producing strains in the hospitals during the strain isolation period. While some of the identified STs were common clinically important STs such as ST11 and ST258 [22–24], some STs were rare, with only a few representative strains reported worldwide (e.g. ST3157 and ST3414 were reported once in Australia (id 6751; isolate DMG1800058) and China (id 7803; isolate 42182), respectively [15]). It is also worth noting that the genomic relatedness among the subspecies of *K. pneumoniae*-type strains is substantially higher than that among *K. quasipneumoniae* subsp., indicating the lack of standardised subspecies delineation criteria among *Klebsiella* spp.

Despite being categorised as carbapenem-resistant, the *K. pneumoniae* strains belonging to ST11 in this study exhibited lower resistance to meropenem compared with other *bla*_{NDM}-harbouring strains from different STs. It is possible the strains from ST11 have a genetic makeup that increases their baseline sensitivity towards meropenem. For example, variations in the gene coding for porin channels have been associated with decreased susceptibility to meropenem, given the direct involvement of such a protein in the permeation of carbapenem [25]. Transposon mutagenesis of these strains followed by selection on higher concentrations of meropenem would be helpful in identifying genes that are associated with increased sensitivity to carbapenem [26,27]. Alternatively, it is also possible that the novel *bla*_{SHV} variants that are present in all other *bla*_{NDM}-containing strains may contribute to the increased resistance towards carbapenem [28].

Most reported carbapenem-resistant Malaysian *K. pneumoniae* strains harbour the *bla*_{NDM1}, *bla*_{OXA-48} or *bla*_{IMP4} gene variant

[29,30]. Isolate MGF020 represents the first *bla*_{KPC-6} harbouring *K. pneumoniae* strain to be isolated in Malaysia. More intriguingly, despite the abundance of *K. pneumoniae* genome assemblies in the NCBI database, contig25 from strain MGF020 is the first *K. pneumoniae* plasmid contig containing the *bla*_{KPC-6} gene. In addition, its significant coverage to the 205 Kb *incA/C* plasmid KP1766_p1 suggests that the *bla*_{KPC-6} gene in MGF020 is harboured on a large *incA/C* plasmid. The putative plasmid length is relatively large compared with most plasmids found in clinical Enterobacteriaceae, which are usually <100Kb [31]. Although a large plasmid can carry more virulence and resistance genes, it also represents a significant burden to the host's metabolism and needs to be maintained in a low copy number. The persistence of large plasmids among *Klebsiella* strains as well as other Gram-negative bacterial genera [32–34], despite the lack of constant selection in the environment, is largely attributed to the presence of an elaborate plasmid-encoded maintenance system [35,36].

The high prevalence of repetitive elements observed in the vicinity of carbapenem resistance genes is the most plausible explanation for the recovery of mostly short *bla* gene-containing contigs from the short-read only assemblies [37]. Such elements are known to break short-read assembly graphs, thus preventing complete assembly of a circular plasmid [38]. The complete assembly of clinically important *Klebsiella* plasmids is highly valuable, as it can provide novel insights into plasmid dynamics and facilitate the tracking of plasmid transmission during outbreaks [39]. Future sequencing work incorporating long reads that can span long repeats, such as those generated by PacBio and Nanopore technology, will be helpful. Nanopore sequencing in particular is now commonly used among researchers to close microbial genome assembly gaps [40,41] due to its ease of use, low capital cost and wide community support.

5. Conclusions

This study provides a significant genomic resource for clinical carbapenem-resistant *K. pneumoniae* isolated from Malaysia. In addition to identifying multiple *K. pneumoniae* sequence types, some of which are rarely reported worldwide, three carbapenem-resistance gene variants were also uncovered that are possibly encoded on different plasmid backbones. Despite the active global genomic sampling of *K. pneumoniae*, this study is the first to report a *K. pneumoniae* plasmid sequence harbouring the *bla*_{KPC-6} gene. These findings highlight the need for increasing the genomic surveillance of clinical *K. pneumoniae* at a national level in view of the emergence of carbapenem-resistant *K. pneumoniae* in this region.

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Competing interests

None.

Ethical approval

Not required.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jgar.2019.07.008>.

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