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Research article

WGS analysis of hypervirulent and MDR *Klebsiella pneumoniae* from Vietnam reveales an inverse relationship between resistome and virulome

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Abstract

The emergence of *Klebsiella (K.) pneumoniae* as a leading cause of nosocomial infections in Southeast Asia is of concern. Vietnam has an outstanding position with regard to antimicrobial-resistant (AMR) pathogens and is a hotspot for carbapenem-resistant *K. pneumoniae*. In the current study, 19 clinical *K. pneumoniae* strains isolated from patients in Vietnam were tested for antibiotic susceptibility, and their genome sequences were analyzed to investigate potential resistance profiles, genotypes, AMR determinants, and virulence-associated genes. More than half of the isolates were multidrug-resistant (MDR), displaying resistance to ciprofloxacin, levofloxacin, chloramphenicol, piperacillin, cefotaxime, ceftazidime, and cefepime. Carbapenem- resistance was detected in 47% (n=9) of isolates. Five isolates were assigned to sequence type (ST) 23 by multi-locus sequence typing (MLST). These ST23 strains exhibited determinants characteristic of hypervirulent strains and were sensitive to all antibiotics tested except for one strain, which was resistant to chloramphenicol and trimethoprim/sulfamethoxazole. All isolates had AMR determinants that conferred resistance to aminoglycosides and ß-lactams. Single nucleotide variants of *oqx*A and *oqx*B conferring resistance to phenicol/quinolone, *omp*K37 and *omp*A conferring resistance to beta-lactams, and *lpt*D conferring resistance to rifamycin, were found in all isolates. Additionally, 95% of the isolates (n=18) carried *fos*A genes; however, only two were resistant to fosfomycin. Five isolates carried genes conferring resistance to colistin; only one carried *mcr*-1.1 and showed resistance to colistin. One hundred thirty virulence-associated genes were identified. The current study demonstrated an inverse relationship between the number of detected virulence determinants and antibiotic resistance. Several well-known resistance genes have been identified but did not mediate resistance, which could be due to insufficient levels or lack of expression. A comprehensive study of the genotypic findings of AMR determinants and virulence with phenotypic data is required. The presence of hypervirulent *K. pneumoniae* carrying specific virulomes is a growing problem, and monitoring of virulence characteristics of *K. pneumoniae* is important.

Keywords: *Klebsiella pneumoniae,* Phenotyping, Hypervirulent MDR, WGS, Resistome, Plasmidome, Virulome, Vietnam

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Introduction

Antimicrobial resistance (AMR) is a major public health problem worldwide with significant health and economic implications, especially in developing and low- and middle-income countries (LMICs) such as the Southeast Asian regions (Gandra et al., 2020). Several multidrug-resistant (MDR) bacterial species exist, and MDR infections with Gram-negative germs are the most common and pose a serious threat to public health (Njeru, 2021; Windham and Kollef, 2022). The highly opportunistic *Klebsiella* (*K.*)

pneumoniae is one of the most common causes of nosocomial infections in Asia and a major agent of extended-spectrum-β-lactamase-producing Enterobacterales (ESBL-E) in Vietnam (Trung et al., 2015) and is generally accompanied by high mortality rates in hospitalized patients. In the last decade, high prevalence of MDR-*K. pneumoniae* (MDR-KP), hypervirulent *K. pneumoniae* (hvKP), and carbapenem-resistant *K. pneumoniae* (CRKP) have been observed in hospitalized patients worldwide and have been associated with unprecedented public health problems in developing and developed countries (Chen et al., 2023; Wareth et al., 2021; Xu et al., 2017). Multidrug-resistant Gram-negative (MRGN) bacteria, particularly ESBL-producing and carbapenem-resistant *Enterobacteriaceae* strains (CRE), are on the rise in intensive care units (ICUs) in Southeast Asia due to a lack of AMR control, accountability as well as limited infection control measures in the health care systems and the communities (Suwantarat and Carroll, 2016). The spread of MDR and CRE is increasing in Vietnamese hospitals (Tran et al., 2019), resulting in a high risk of treatment failures with significant negative economic impacts and ultimately death. Additionally, the incidence of MDR-KP, CRKP, and hvKP is increasing in intensive care units in Vietnam (Breurec et al., 2013; Sewunet et al., 2022; Tran et al., 2019).

Thus, the current study aimed to phenotypically and genetically characterize AMR determinants, pathogenicity-associated genes, and plasmid replicons in *K. pneumoniae* strains obtained from clinical samples in Hanoi. Next-generation sequencing (NGS) technology was used to investigate the relationship between the resistome and the virulome in these clinical isolates.

Material and Methods

Bacterial isolates, identification, and antibiotic susceptibility testing (AST)

Nineteen *K. pneumoniae* isolates were obtained from clinical samples from Vietnamese patients at the General Hospital of Phutho, Hanoi. Strains were isolated from blood (n=7), sputum (n=3), urine (n=4), abscess specimens (n=3), and phlegm samples (n=2) of patients admitted to the hospital in 2017. The strains were sent to the Institute of Bacterial Infections and Zoonoses (IBIZ, Jena, Germany) for species confirmation and typing. All isolates were identified by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS; Bruker, Germany) with a log value > 2,300 (Khater et al., 2021).

The minimum inhibitory concentration (MIC) was determined by the broth microdilution method as previously described (Wareth et al., 2020), using the automated MICRONAUT-S system (Micronaut, MERLIN Diagnostics GmbH, Bornheim-Hersel Germany). The sensitivity of the isolates was determined for a panel of 18 antibiotics: ciprofloxacin (CIP), levofloxacin (LEV), amikacin (AMK), chloramphenicol (CMP), fosfomycin (FOS), tigecycline (TGC), colistin (COL), trimethoprim/sulfamethoxazole (T/S), piperacillin (PIP), piperacillin/tazobactam (PIT), cefotaxime (CTX), ceftazidime (CAZ), ceftazidime/avibactam (CAA), ceftolozane/tazobactam (CTA), cefepime (CEP), imipenem (IMP), meropenem (MER) and ertapenem (ERT). The results were automatically evaluated with the MICRONAUT-S software, based on the MIC values of the Clinical and Laboratory Standards Institute (CLSI) breakpoint guidelines available for *K. pneumoniae* as susceptible, intermediate, and resistant.

Ethical statement

The current study did not require ethical approval since it only involved bacterial isolates that were already stored in the microbiology laboratory of the General Hospital of Phutho, Hanoi. Patient specimens or data were not analyzed. In accordance with the Nagoya Protocol, we obtained consent from the Ministry of Natural Resources and Environment, under reference Nr. 1435/QD-BTNMT, to receive the genetic samples.

Whole genome sequencing and *in-silico* detection of AMR determinants, virulome genes, and the plasmidome

Genomic DNA was extracted from fresh colonies on blood agar using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Sequencing libraries were prepared with the Nextera XT DNA Library Preparation Kit (Illumina, Inc., San Diego, CA, USA) and subsequently submitted to paired-end sequencing on an Illumina MiSeq sequencer (Illumina Inc., San Diego, CA, USA). Analysis and quality control of the raw sequencing data and quality checks of the assembled genomes were performed as previously described (Linde et al., 2020; Wareth et al., 2020). Kraken2 v2.0.7 beta (Wood et al., 2019) was used to confirm the identity of the strains. The databases Comprehensive Antibiotic Resistance Database (CARD) (Jia et al., 2017), ResFinder (Zankari et al., 2012), and NCBI's AMRFinderPlus tool (Feldgarden et al., 2019) were applied to determine the genetic features leading to AMR. The PlasmidFinder database (Carattoli et al., 2014) was used to identify present plasmid replicons. *In-silico* determination of classical multilocus sequence typing (MLST) was done using the in-house pipeline WGSBAC (https://gitlab.com/FLI_Bi

oinfo/WGSBAC) and the software mlst v2.16.1 (https://github.com/tseemann/mlst) that uses the PubMLST website (Jolley and Maiden, 2010) and the scheme proposed by Diancourt and colleagues (Diancourt et al., 2005). The Virulence Factor Database (Chen et al., 2005) was used to predict virulence-associated genes. Single-nucleotide polymorphisms (SNPs) were determined using Snippy v4.6.0 (https://github.com/tseemann/snippy) with *K. pneumoniae* strain ED23 (GCF_001708225.1) as the reference genome. The resulting core genome SNP (cgSNP) alignment was used for phylogenetic reconstruction with RAxML v8.2.12 (Stamatakis, 2014) by applying the maximum likelihood method. The tree was visualized with Microreact (Argimón et al., 2016).

Results

Whole genome sequencing (WGS) data and MLST analysis

A total of 20 isolates were analyzed. WGS data using the Kraken2 program confirmed 19 isolates as *K. pneumoniae,* and one isolate obtained from phlegm was identified as *K. quasipneumoniae* and thus excluded from the subsequent analyses. Genome sequencing of the 19 confirmed *K. pneumoniae* isolates yielded an average number of 1,937,703 reads per isolate (range 1,420,128 – 2,989,006). The isolates' mean coverage was 89-fold (range: 65-fold to 143-fold). Genome assembly yielded genome sizes between 5,146,468 bp and 5,904,625 bp. The GC content was, on average, 57.2%. The mean N50 of the 19 assembled genomes was 284,376 bp (range 173,054 bp – 441,209 bp). MLST analyses of the 19 *K. pneumoniae* strains allocated all the strains into a distinct sequence type (ST). Five strains were assigned to ST23, three to ST16, two isolates each to ST15 and ST11, and one isolate each to ST2856, ST709, ST592, ST395, ST367, ST268 and ST45 (Table 1). The cgSNP analysis of the 19 isolates agreed with the MLST result, yielding clusters according to the STs (Figure 1).

Phenotyping and AMR determinants in *K. pneumoniae* isolates

Antibiotic susceptibility testing of the 19 *K. pneumoniae* strains revealed that more than half of the isolates were MDR, showing resistance to more than three antimicrobial groups. 12 (63%) isolates displayed resistance to piperacillin (penicillins), 11 (58%) isolates were resistant to ciprofloxacin (fluoroquinolones), chloramphenicol (amphenicol), cefotaxime and ceftazidime (3rd generation cephalosporins), and 10 (53%) isolates were resistant to levofloxacin (fluoroquinolones) and cefepime (4th generation cephalosporins). Additionally, nine (47%) isolates were resistant to trimethoprim/sulfamethoxazol (sulfonamides) and ceftolozan/tazobactam. Non-susceptibility (resistance and susceptibility with increased doses) to carbapenem compounds was seen in more than one-third of isolates. Nine (47%) isolates showed resistance to ertapenem and seven (37%) to imipenem and meropenem. The highest susceptibility was seen to colistin and tigecycline, followed by fosfomycin (Table 1). Non-susceptibility to colistin and tigecycline was seen only once in an isolate obtained from an abscess, while resistance to fosfomycin was seen in two isolates obtained from sputum and an abscess. Six isolates were susceptible to all tested antibiotics, among them four belonging to ST23 (which was classified as hvKP), one belonging to ST592, and one to ST367 (Table 1). Another ST23 isolate displayed resistance only to chloramphenicol and trimethoprim/sulfamethoxazole and harboured *cml*A1 and *sul*3.

A wide variety of resistance genes, conferring resistance mainly to aminoglycosides (n=17), beta-lactams (n=32), tetracycline (*tet.* A, *tet.*D, *tmex*C1, *tmex*D1 and *topr*J1), fosfomycin (*fos*A variants), sulfonamides (*sul*1, *sul*2 and *sul*3), rifampicin, phenicol and quinolones, macrolides, diaminopyrimidine, and colistin were determined *in silico*. Isolate Viet-Hano7 (ST709) obtained from an abscess harboured the highest number of AMR determinants (n=47), followed by two isolates obtained from blood and abscess that belonged to ST11 and harboured 46 and 45 AMR genes, respectively. The lowest number of AMR genes was found in isolates obtained from blood assigned to ST592 (n=16 genes), ST2856 (n=17 genes), and four isolates belonging to ST23 (n=18 genes). Quantitive evaluation of resistance genes was directly proportional to susceptibility. The susceptible isolates harboured fewer resistance genes, whereas the number of AMR genes was inversely proportional to the number of virulence genes determined, i.e., isolates that harbored higher numbers of AMR genes carried fewer virulence-associated determinants (Table 1). All strains, whether resistant or susceptible, harboured at least one resistance gene variant conferring resistance to beta-lactams, i.e., *Omp*K37 and *Omp*A, to fosfomycin, i.e., *fos*A variants, to rifampicin, i.e., *Lpt*D, to phenicol, and quinolone, i.e., *oqx*A and *oqx*B variants (Figure 1). The *fos*A variants conferring resistance to fosfomycin were present in all isolates, although only two isolates displayed

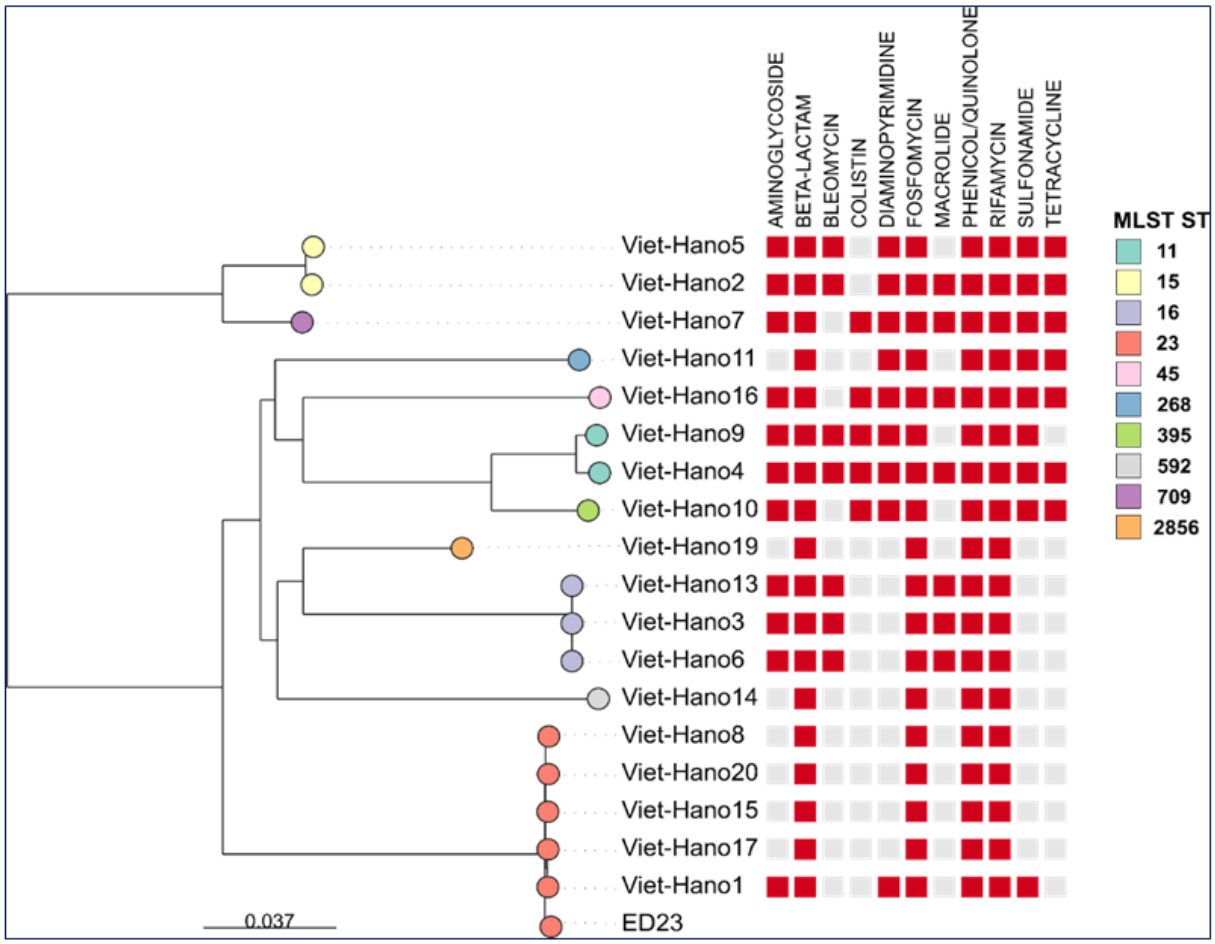

Figure 1: Maximum likelihood tree generated from cgSNP alignment of *K. pneumonia* isolates from Vietnam using *K. pneumoniae* strain ED23 as reference. Leaf colors show the MLST sequence type. To the right of the isolate names, the *in silico* resistance profile of the major antibiotic groups is shown (red: resistance genes detected; grey: no detected resistance genes). The bar at the bottom indicates the number of base substitutions per site.

resistance in the sensitivity testing. Multidrug resistance genes, i.e., *ram*A, *acr*A, *kpn*E, *kpn*F, *kpn*G, and *kpn*H, were identified in almost all isolates.

Nine (47%) isolates displayed resistance to sulfonamides, all of which harboured either *sul*1, *sul*2, or *sul*3 genes. Seven (37%) isolates displayed resistance to amikacin and harboured *aac*(6')-Ib-cr5 conferring resistance to aminoglycosides, e.g., amikacin/kanamycin/quinolone/tobramycin. In contrast, two isolates were susceptible to amikacin, despite harbouring several AMR genes theoretically conferring resistance to aminoglycosides, i.e., *aac*(6')-Ib-cr5, *aph*(6)-Id, *aph*(3'')-Ib, *ant*(3'')-IIa, *ant*(3'')-Ia, *aph*(3')-Ia, *aad*A2, *ant*(2'')-Ia and *aac*(3)-IId. In the same context, one isolate displayed resistance to colistin and harboured *mcr*-1.1 genes, while four isolates harboured a well-known, potentially resistance-conferring point mutation in the colistin-resistance gene *pmr*B resulting in an amino acid substitution (R256G) despite being susceptible to colistin.

Characterization of plasmid replicons and virulence-associated genes in *K. pneumoniae*

PlasmidFinder was used to identify potential plasmid replicons in the assemblies. This analysis revealed 25 different types of plasmid replicons in the 19 *K. pneumoniae* isolates. All isolates were found to carry plasmid replicons. However, the number of replicons varied between the strains, ranging from two to nine. Seven replicons belonged to Col-type plasmids, of which Col.pHAD28.1 was the most predominant and found in eight isolates. Nine replicons belonged to the IncF plasmid family, of which IncFIB.K._1 was the most prevalent, identified in nine isolates. Six replicons belonged to the Inc-type plasmid family, of which IncHI1B.pNDM.MAR.1 was the most predominant in eight isolates. Additionally, plasmid replicons p0111_1, repB.R1701.1, and repB_KLEB_VIR were found in one, three, and eight isolates, respectively. Four strains carried the highest number of plasmid replicons (n = 9). These contained a low number of virulence-associated genes (n = 63). In contrast, isolates that carried the lowest number

Table 1: Antibiotic susceptibility of *K. pneumonia* isolates from Vietnam and their MLST sequence types. S – susceptible; R – resistant; I – intermediate resistance, according to CLSI breakpoint values in comparison to the number of detected AMR and virulence genes. The isolates are arranged according to the number of identified AMR genes in ascending order. Yellow marks the resistance profiles of MLST ST23 isolates.

No. detected AMR genes	No. detected virulence genes	MLST	CIP	LEV	AMK	COL	CMP	FOS	TGC	T/S	PIP	PIT	CTX	CAZ	CAA	CTA	IMP	MER	ERT	CEP	ID
16	72	592	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	Viet-Hano14
17	75	2856	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	Viet-Hano19
18	130	23	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	Viet-Hano8
18	130	23	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	Viet-Hano18
18	126	23	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	Viet-Hano16
18	128	23	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	Viet-Hano20
18	74	367	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	Viet-Hano13
21	107	268	R	R	S	S	R	S	I	R	R	I	R	R	S	R	S	S	I	S	Viet-Hano11
26	129	23	S	S	S	S	R	S	S	R	S	S	S	S	S	S	S	S	S	S	Viet-Hano1
30	63	16	R	R	R	S	I	S	S	S	R	R	R	R	R	R	R	R	R	R	Viet-Hano3
30	63	16	R	R	R	S	I	S	S	S	R	R	R	R	R	R	R	R	R	R	Viet-Hano6
30	63	16	R	R	R	S	I	S	S	S	R	R	R	R	R	R	R	R	R	R	Viet-Hano13
34	69	395	R	R	R	S	R	S	S	R	R	S	R	R	S	S	S	S	S	R	Viet-Hano10
37	71	15	R	R	R	S	S	R	S	R	R	I	R	R	R	R	I	R	R	R	Viet-Hano5
37	87	45	I	S	S	S	R	S	S	R	R	S	R	R	S	I	S	S	I	I	Viet-Hano16
44	80	15	R	R	S	S	R	S	S	R	R	S	R	R	R	R	S	R	R	R	Viet-Hano2
45	67	11	R	R	S	S	R	R	S	R	R	R	R	R	R	R	R	R	R	R	Viet-Hano9
46	71	11	R	R	R	S	R	S	S	R	R	R	R	R	R	R	R	R	R	R	Viet-Hano4
47	67	709	R	R	R	R	R	S	S	R	R	S	R	R	S	S	I	S	S	R	Viet-Hano7
Total number of non-susceptible			11	10	7	1	11	2	1	9	12	7	11	11	7	9	7	7	9	10	

Ciprofloxacin (CIP), levofloxacin (LEV), amikacin (AMK), chloramphenicol (CMP), fosfomycin (FOS), tigecycline (TGC), colistin (COL), trimethoprim/sulfamethoxazole (T/S), piperacillin (PIP), piperacillin/tazobactam (PIT), cefotaxime (CTX), ceftazidime (CAZ), ceftazidime/avibactam (CAA), ceftolozane/tazobactam (CTA), cefepime (CEP), imipenem (IMP), meropenem (MER) and ertapenem (ERT).

of plasmid replicons (n = 2) contained the highest number of virulence-associated genes (n = 74-130). ST23 isolates harboured the lowest number of replicons (2-3 replicons), i.e., IncFIB.pKPHS1.1, IncHI1B.pNDM.MAR.1, IncI.Gamma.1 and repB_KLEB_VIR. The most abundant plasmid replicons identified belonged to the IncF plasmid family (IncFIB.K._1 in 9 isolates), Col family (Col.pHAD28.1 in 8 isolates), and Inc-like (IncHI1B.pNDM.MAR.1 in 8 isolates).

In the current set of isolates, 130 virulence-associated genes were identified. The numbers of identified virulence genes were inversely proportional to the number of AMR genes determined. Five ST23 isolates harboured the highest number of virulence-associated genes, ranging between 126 and 130, followed by an ST268 isolate that harboured 107 genes. All six isolates harboured determinants discriminatory for hypervirulent *K. pneumoniae* (hvKp), which mostly influenced iron uptake, e.g., the prototypic catecholate siderophore enterobactins (*ent*A, *ent*B, *fep*C), the citrate-hydroxamate siderophore aerobactins (*iuc*A-D, *iut*A), four glycosylated salmochelins (*iro*B, *iro*C, *iro*D, *iro*N), the regulators of mucoid phenotype A (*rmp*A2, *rmp*A), the siderophore yersiniabactin-related genes (*ybt*A, *ybt*E, *ybt*P, *ybt*Q, *ybt*S, *ybt*T, *ybt*U, *ybt*X, *irp*1, *irp*2, *fyu*A), and the colibactin exotoxin genes *clb*A-Q, and *clb*S. Additional genes that were identified predominantly in ST23 isolates were *vgr*G/*tss*I, which is related to the T6SS secretion system, eight immune modulation regulators, and anti-phagocytosis capsular factors KP1_RS variants (KP1_RS17295, KP1_RS17300, KP1_RS17305, KP1_RS17315, KP1_RS17320, KP1_RS17325, KP1_RS17330, and KP1_RS17335). The allantoin utilization genes *all*A-D and *all*R-S associated with hypervirulent *K. pneumoniae* strains were exclusively found in ST23 isolates.

Discussion

The spread of MDR and CRKP in the healthcare system throughout the world has become a serious public health problem, as it causes a wide range of severe infections and high mortality rates (Lee et al., 2023; Wareth et al., 2021). Southeast Asian countries, especially Vietnam, are hotspots for AMR. Several resistant ESKAPE pathogens, such as *S. aureus, K. pneumoniae, A. baumannii,* and *P. aeruginosa,* have been isolated from inpatients with bloodstream infections in Vietnam (Van An et al., 2023). In the present study, 19 *K. pneumoniae* isolates were recovered from blood, sputum, urine, abscess, and phlegm samples collected from Vietnamese patients admitted to a hospital in Hanoi. More than half of the isolates were MDR and harboured resistance genes conferring resistance to penicillins, fluoroquinolones, chloramphenicol, 3rd and 4th generation cephalosporins, and sulphonamides. However, the isolates were still susceptible to colistin, tigecycline, and fosfomycin. Previously, a combination of colistin, tigecycline, and fosfomycin has shown a synergetic effect against ESBL-producing KP infections in Taiwan (Ku et al., 2017), which is consistent with our results that these three antibiotics could be considered as the last-resort treatment to MDR *K. pneumoniae*, particularly CRKP isolates from Southeast Asia. Combining colistin or fosfomycin with other antibiotic compounds has been recommended against MDR *K. pneumoniae* strains in different regions of the world (Evren et al., 2013; Fan et al., 2016; Ku et al., 2017). Around half of the isolates in the present study displayed resistance to carbapenem compounds. The rapid spread of CPKP poses a serious threat to both patients and public health in Vietnam. The prevalence of carbapenem-resistant *Enterobacteriaceae* has been shown to be high among humans and animals in the Mekong Delta (Yen et al., 2022), highlighting the potential transmission of these carbapenem-resistant bacteria between hospitals and community settings.

Five of the 19 isolates were assigned to ST23. These isolates harboured the highest number of virulence-associated genes, such as the discriminatory determinants for hypervirulent *K. pneumoniae* (hvKp), particularly the allantoin utilization genes *all*A-D and *all*R-S that have been associated with hvKp strains (Cusa et al., 1999), as well as aerobactin, yersiniabactin, salmochelin, and hypermucoid locus (*rmp*A) genes that are associated with severe infections in humans (Sumbana et al., 2023). Despite the hvKp isolates in the current study possessing more virulence factors than the other "classical" *K. pneumoniae* strains, all of the hvKp isolates were susceptible to all tested antibiotics, including carbapenems and harbored a lower number of AMR genes. This is consistent with other studies that showed that hvKP isolates are susceptible to most antibiotics (Surgers et al., 2016); this can be explained by rapid intervention in these cases with appropriate therapy due to severe clinical signs or the death of patients before treatment completion. The hvKP is being increasingly reported globally and poses a major clinical and public health threat (Huang et al., 2023).

In the current study, genes conferring resistance to ß-lactams, such as *omp*K37 and *omp*A, were found in all isolates, while *bla*$_{SHV}$ variants were found in 18 of the 19 isolates. Only one strain (ST367) was devoid of *bla*$_{SHV}$ and susceptible to all tested antibiotics. The *bla*$_{SHV}$ variants are among the most frequently detected genes in *K. pneumoniae* (Wareth et al., 2021). The presence of the potentially resistance-conferring substitution R256G in PmrB in four isolates did

not confer colistin resistance, although resistance to colistin in *K. pneumoniae* has been attributed to mutations in *pmr*AB genes (Nirwan et al., 2021). Therefore, the presence of mutations in this gene does not necessarily result in the development of resistance. Alterations in the chromosomal coding regions of this regulatory gene were found to be required to obtain a colistin-resistant phenotype (Bolourchi et al., 2021). Six isolates (four ST23, one ST592, and one ST367) were susceptible to all tested antibiotics, although they harboured at least 16 AMR-associated genes. For example, *fos*A failed to mediate resistance to fosfomycin in 17 isolates in the current *K. pneumoniae* isolates, and two isolates were susceptible to amikacin despite harbouring several AMR genes with the potential of conferring resistance to aminoglycosides. The presence of specific genes in the bacterial genome does not necessarily induce their translation and function. Some genes are active; some are switched off (Wareth et al., 2017). This occurrence can also be considered in the case of AMR genes. A sufficient gene translation and protein expression level is required to enable a resistance determinants function. Expression level and timing can significantly affect gene functions, as insufficient expression levels will not mediate resistance (Yuan-Chuan, 2020). Conversely, the presence of a molecule inhibitor, as in the case of *fos*A, could restore the activity of fosfomycin or significantly potentiate fosfomycin activity against pathogens harbouring the *fos*A gene (Tomich et al., 2019).

Several virulence genes were identified among the isolates (range 63 – 129). Five ST23 strains harboured virulence genes that contribute to the hypervirulent phenotype, including the allantoin utilization genes *all*A-D and *all*R-S (Cusa et al., 1999), the iron uptake aerobactins which are considered the major hvKp-specific virulence factors (Liu and Guo, 2019; Russo and Gulick, 2019), and the major regulator of the mucoid phenotype *K. pneumoniae* (*rmpA*/*A2*) (Catalán-Nájera et al., 2017; Zhang et al., 2016). The *wca*J gene, which acts as an initiation glycosyltransferase gene in capsular polysaccharide (CPS) synthesis, was found in all ST23 isolates. Over-expression of *wca*J was associated with increased viscosity, while its knockout led to the loss of the capsule in *K. pneumoniae* (Wang et al., 2023). The hvKp strains have recently elicited concern because they can cause serious invasive illnesses. The presence of such isolates among patients is alarming and requires further investigation.

Plasmids are a major factor contributing to the dissemination of resistance and virulence-associated genes in bacteria. Investigation of plasmids and their replicons revealed the presence of a diverse replicon content in the current set of isolates (range 2-9 replicons). The most abundant plasmid replicons identified belonged to the IncF plasmid family (IncFIB.K._1), the Col family (Col.pHAD28.1), and the Inc-like family (IncHI1B.pNDM.MAR.1). Ten isolates harboured IncF plasmids, which have been associated with the emergence of $bla_{\text{CTX-M-15}}$ globally (Ho et al., 2015; Villa et al., 2010); however, only six isolates harboured $bla_{\text{CTX-M-15}}$, and one isolate harboured $bla_{\text{CTX-M-27}}$. ST23 isolates showed the lowest number of plasmid replicons (2 or 3 replicons). IncHI1B.pNDM.MAR.1 and repB_KLEB_VIR were identified in all ST23 isolates. The repB_KLEB_VIR plasmid has always been found in hvKP isolates. It is known to carry aerobactin and salmochelin virulence genes (Muraya et al., 2022). Concurrently, ST16 isolates harboured the highest number of replicons (9 replicons) and the same AMR and virulence-associated genes.

The present study demonstrated that the hvKP isolates were susceptible to all tested antibiotics and had fewer AMR genes despite containing only two plasmid replicons and the highest number of virulence-associated genes, showing an inverse relationship between the number of detected virulence determinants and antibiotic resistance genes. This may be due to a severe course of infection resulting in the death of patients or to the correct treatment of patients and the prevention of AMR development.

Further studies with a large set of isolates should be conducted to comprehensively investigate the correlation between the genotypic findings of AMR and virulence with phenotypic data. Despite hvKP isolates being susceptible to all tested antibiotics, there is a growing global concern about its emergence in healthcare systems and non-human sources (Banerjee et al., 2021; Mario et al., 2023) due to severe infections occurring in patients with potential treatment failure and possible transmission to animals and the environment. Thus, monitoring virulence characteristics of *K. pneumoniae* from a One Health perspective in Vietnam and neighbouring AMR hot spots in southeast Asian regions is of utter importance.

Article Information

Conflict of Interest. The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Argimón, S., Abudahab, K., Goater, R.J.E., Fedosejev, A., Bhai, J., Glasner, C., Feil, E.J., Holden, M.T.G., Yeats, C.A., Grundmann, H., Spratt, B.G., Aanensen, D.M., 2016. Microreact: visualizing and sharing data for genomic epidemiology and phylogeography. Microbial Genomics 2, e000093. https://doi.org/10.1099/mgen.0.000093.

Banerjee, T., Wangkheimayum, J., Sharma, S., Kumar, A., Bhattacharjee, A., 2021. Extensively drug-resistant hypervirulent *Klebsiella pneumoniae* from a series of neonatal sepsis in a tertiary care hospital, India. Frontiers in Medicine 8, 645955. https://doi.org/10.3389/fmed.2021.645955

Bolourchi, N., Shahcheraghi, F., Giske, C.G., Nematzadeh, S., Noori Goodarzi, N., Solgi, H., Badmasti, F., 2021. Comparative genome analysis of colistin-resistant OXA-48-producing *Klebsiella pneumoniae* clinical strains isolated from two Iranian hospitals. Annals of Clinical Microbiology and Antimicrobials 20, 74. https://doi.org/10.1186/s12941-021-00479-y

Breurec, S., Guessennd, N., Timinouni, M., Le, T.A., Cao, V., Ngandjio, A., Randrianirina, F., Thiberge, J.M., Kinana, A., Dufougeray, A., Perrier-Gros-Claude, J.D., Boisier, P., Garin, B., Brisse, S., 2013. *Klebsiella pneumoniae* resistant to third-generation cephalosporins in five African and two Vietnamese major towns: multiclonal population structure with two major international clonal groups, CG15 and CG258. Clinical Microbiology and Infection 19, 349-355. https://doi.org/10.1111/j.1469-0691.2012.03805.x

Carattoli, A., Zankari, E., García-Fernández, A., Voldby Larsen, M., Lund, O., Villa, L., Møller Aarestrup, F., Hasman, H., 2014. *In silico* detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Antimicrobial Agents and Chemotherapy 58, 3895-3903. https://doi.org/10.1128/aac.02412-14.

Catalán-Nájera, J.C., Garza-Ramos, U., Barrios-Camacho, H., 2017. Hypervirulence and hypermucoviscosity: Two different but complementary *Klebsiella* spp. phenotypes? Virulence 8, 1111-1123. https://doi.org/10.1080/21505594.2017.1317412

Chen, J., Zhang, H., Liao, X., 2023. Hypervirulent *Klebsiella pneumoniae.* Infection and Drug Resistance 16, 5243-5249. https://doi.org/10.2147/idr.S418523

Chen, L., Yang, J., Yu, J., Yao, Z., Sun, L., Shen, Y., Jin, Q., 2005. VFDB: a reference database for bacterial virulence factors. Nucleic Acids Research 33, D325-328. https://doi.org/10.1093/nar/gki008

Cusa, E., Obradors, N., Baldomà, L., Badía, J., Aguilar, J., 1999. Genetic analysis of a chromosomal region containing genes required for assimilation of allantoin nitrogen and linked glyoxylate metabolism in *Escherichia coli*. Journal of Bacteriology 181, 7479-7484. https://doi.org/10.1128/jb.181.24.7479-7484.1999.

Diancourt, L., Passet, V., Verhoef, J., Grimont, P.A., Brisse, S., 2005. Multilocus sequence typing of *Klebsiella pneumoniae* nosocomial isolates. Journal of Clinical Microbiology 43, 4178-4182. https://doi.org/10.1128/jcm.43.8.4178-4182.2005.

Evren, E., Azap, O.K., Çolakoğlu, Ş., Arslan, H., 2013. In vitro activity of fosfomycin in combination with imipenem, meropenem, colistin, and tigecycline against OXA 48-positive *Klebsiella pneumoniae* strains. Diagnostic Microbiology and Infectious Disease 76, 335-338. https://doi.org/10.1016/j.diagmicrobio.2013.04.004.

Fan, B., Guan, J., Wang, X., Cong, Y., 2016. Activity of colistin in combination with meropenem, tigecycline, fosfomycin, fusidic acid, rifampin or sulbactam against extensively drug-resistant *Acinetobacter baumannii* in a murine thigh-infection model. PLoS One 11, e0157757. https://doi.org/10.1371/journal.pone.0157757.

Feldgarden, M., Brover, V., Haft, D.H., Prasad, A.B., Slotta, D.J., Tolstoy, I., Tyson, G.H., Zhao, S., Hsu, C.H., McDermott, P.F., Tadesse, D.A., Morales, C., Simmons, M., Tillman, G., Wasilenko, J., Folster, J.P., Klimke, W., 2019. Validating the AMRFinder tool and resistance gene database by using antimicrobial resistance genotype-phenotype correlations in a collection of isolates. Antimicrobial Agents and Chemotherapy 63. https://doi.org/10.1128/aac.00483-19.

Gandra, S., Alvarez-Uria, G., Turner, P., Joshi, J., Limmathurotsakul, D., van Doorn, H.R., 2020. Antimicrobial resistance surveillance in low- and middle-income countries: Progress and challenges in eight South Asian and Southeast Asian Countries. Clinical Microbiology Reviews 33. https://doi.org/10.1128 /cmr.00048-19.

Ho, W.S., Yap, K.P., Yeo, C.C., Rajasekaram, G., Thong, K.L., 2015. 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. Frontiers in Microbiology 6, 1547. https://doi.org/10.3389/fmicb.2015.01547.

Huang, L., Li, Y., Xu, C., Zhou, M., Wang, T., Wang, T., Wang, J., Tang, J., Li, Y., Dong, N., 2023. A novel virulence plasmid encoding yersiniabactin, salmochelin, and RmpADC from hypervirulent *Klebsiella pneumoniae* of distinct genetic backgrounds. Antimicrobial Agents and Chemotherapy, e0093523. https://doi.org/10.1128/aac.00935-23.

Jia, B., Raphenya, A.R., Alcock, B., Waglechner, N., Guo, P., Tsang, K.K., Lago, B.A., Dave, B.M., Pereira, S., Sharma, A.N., Doshi, S., Courtot, M., Lo, R., Williams, L.E., Frye, J.G., Elsayegh, T., Sardar, D., Westman, E.L., Pawlowski, A.C., Johnson, T.A., Brinkman, F.S., Wright, G.D., McArthur, A.G., 2017. CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. Nucleic acids research 45, D566-d573. https://doi.org/10.1093/nar/gkw1004.

Jolley, K.A., Maiden, M.C., 2010. BIGSdb: Scalable analysis of bacterial genome variation at the population level. BMC Bioinformatics 11, 595. https://doi.org/10.1186/1471-2105-11-595.

Khater, D.F., Lela, R.A., El-Diasty, M., Moustafa, S.A., Wareth, G., 2021. Detection of harmful foodborne pathogens in food samples at the points of sale by MALDT-TOF MS in Egypt. BMC Research Notes 14, 112. https://doi.org/10.1186/s13104-021-05533-8.

Ku, Y.-H., Chen, C.-C., Lee, M.-F., Chuang, Y.-C., Tang, H.-J., Yu, W.-L., 2017. Comparison of synergism between colistin, fosfomycin, and tigecycline against extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* isolates or with carbapenem resistance. Journal of Microbiology, Immunology and Infection 50, 931-939. https://doi.org/10.1016/j.jmii.2016.12.008.

Lee, J., Sunny, S., Nazarian, E., Fornek, M., Abdallah, M., Episcopia, B., Rowlinson, M.C., Quale, J., 2023. Carbapenem-Resistant *Klebsiella pneumoniae* in Large

Public Acute-Care Healthcare System, New York, New York, USA, 2016-2022. Emerging Infectious Diseases 29, 1973-1978. https://doi.org/10.3201/eid2910.230153.

Linde, J., Homeier-Bachmann, T., Dangel, A., Riehm, J.M., Sundell, D., Öhrman, C., Forsman, M., Tomaso, H., 2020. Genotyping of *Francisella tularensis* subsp. holarctica from hares in Germany. Microorganisms 8. https://doi.org/10.3390/microorganisms8121932.

Liu, C., Guo, J., 2019. Hypervirulent *Klebsiella pneumoniae* (hypermucoviscous and aerobactin positive) infection over 6 years in the elderly in China: antimicrobial resistance patterns, molecular epidemiology and risk factor. Annals of Clinical Microbiology and Antimicrobials 18, 4. https://doi.org/10.1186/s12941-018-0302-9.

Mario, E., Hamza, D., Abdel-Moein, K., 2023. Hypervirulent *Klebsiella pneumoniae* among diarrheic farm animals: A serious public health concern. Comparative Immunology, Microbiology and Infectious Diseases 102, 102077. https://doi.org/10.1016/j.cimid.2023.102077.

Muraya, A., Kyany'a, C., Kiyaga, S., Smith, H.J., Kibet, C., Martin, M.J., Kimani, J., Musila, L., 2022. Antimicrobial resistance and virulence characteristics of *Klebsiella pneumoniae* isolates in Kenya by whole-genome sequencing. Pathogens 11. https://doi.org/10.3390/pathogens11050545.

Nirwan, P.K., Chatterjee, N., Panwar, R., Dudeja, M., Jaggi, N., 2021. Mutations in two-component systems (PhoPQ and PmrAB) in colistin-resistant *Klebsiella pneumoniae* from North Indian tertiary care hospital. The Journal of Antibiotics 74, 450-457. https://doi.org/10.1038/s41429-021-00417-2.

Njeru, J., 2021. Emerging carbapenem resistance in ESKAPE pathogens in sub-Saharan Africa and the way forward. German Journal of Microbiology 1, 3-6. https://doi.org/10.51585/gjm.52021.50002.

Russo, T.A., Gulick, A.M., 2019. Aerobactin Synthesis Proteins as Antivirulence Targets in Hypervirulent *Klebsiella pneumoniae*. ACS Infectious Diseases 5, 1052-1054. https://doi.org/10.1021/acsinfecdis.9b00117.

Sewunet, T., K, K.S., Nguyen, H.H., Sithivong, N., Hoang, N.T.B., Sychareun, V., Nengmongvang, K., Larsson, M., Olson, L., Westerlund, F., Giske, C.G., 2022. Fecal carriage and clonal dissemination of blaNDM-1 carrying *Klebsiella pneumoniae* sequence type 147 at an intensive care unit in Lao PDR. PLoS One 17, e0274419. https://doi.org/10.1371/journal.pone.0274419.

Stamatakis, A., 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30, 1312-1313. https://doi.org/10.1093/bioinformatics/btu033.

Sumbana, J.J., Santona, A., Abdelmalek, N., Fiamma, M., Deligios, M., Manjate, A., Sacarlal, J., Rubino, S., Paglietti, B., 2023. Polyclonal multidrug ESBL-producing *Klebsiella pneumoniae* and emergence of susceptible hypervirulent *Klebsiella pneumoniae* ST23 isolates in Mozambique. Antibiotics (Basel) 12. https://doi.org/10.3390/antibiotics12091439.

Surgers, L., Boyd, A., Girard, P.M., Arlet, G., Decré, D., 2016. ESBL-producing strain of hypervirulent *Klebsiella pneumoniae* K2, France. Emerging Infectious Diseases 22, 1687-1688. https://doi.org/10.3201/eid2209.160681.

Suwantarat, N., Carroll, K.C., 2016. Epidemiology and molecular characterization of multidrug-resistant Gram-negative bacteria in Southeast Asia. Antimicrobial Resistance and Infection Control 5, 15. https://doi.org/10.1186/s13756-016-0115-6.

Tomich, A.D., Klontz, E.H., Deredge, D., Barnard, J.P., McElheny, C.L., Eshbach, M.L., Weisz, O.A., Wintrode, P., Doi, Y., Sundberg, E.J., Sluis-Cremer, N., 2019. Small-molecule inhibitor of *fos*A expands fosfomycin activity to multidrug-resistant Gram-negative pathogens. Antimicrobial Agents and Chemotherapy 63, e01524-01518. https://doi.org/10.1128/AAC.01524-18.

Tran, D.M., Larsson, M., Olson, L., Hoang, N.T.B., Le, N.K., Khu, D.T.K., Nguyen, H.D., Vu, T.V., Trinh, T.H., Le, T.Q., Phan, P.T.T., Nguyen, B.G., Pham, N.H., Mai, B.H., Nguyen, T.V., Nguyen, P.T.K., Le, N.D., Huynh, T.M., Anh Thu, L.T., Thanh, T.C., Berglund, B., Nilsson, L.E., Bornefall, E., Song, L.H., Hanberger, H., 2019. High prevalence of colonization with carbapenem-resistant *Enterobacteriaceae* among patients admitted to Vietnamese hospitals: Risk factors and burden of disease. Journal of Infection 79, 115-122. https://doi.org/10.1016/j.jinf.2019.05.013.

Trung, N.T., Hien, T.T., Huyen, T.T., Quyen, D.T., Binh, M.T., Hoan, P.Q., Meyer, C.G., Velavan, T.P., Song le, H., 2015. Simple multiplex PCR assays to detect common pathogens and associated genes encoding for acquired extended spectrum betalactamases (ESBL) or carbapenemases from surgical site specimens in Vietnam. Annals of Clinical Microbiology and Antimicrobials 14, 23. https://doi.org/10.1186/s12941-015-0079-z

Van An, N., Hoang, L.H., Le, H.H.L., Thai Son, N., Hong, L.T., Viet, T.T., Le, T.D., Thang, T.B., Vu, L.H., Nguyen, V.T.H., Xuan Nguyen, K., 2023. Distribution and antibiotic resistance characteristics of bacteria isolated from blood culture in a teaching hospital in Vietnam during 2014-2021. Infection and Drug Resistance 16, 1677-1692. https://doi.org/10.2147/idr.S402278.

Villa, L., García-Fernández, A., Fortini, D., Carattoli, A., 2010. Replicon sequence typing of IncF plasmids carrying virulence and resistance determinants. Journal of Antimicrobial Chemotherapy 65, 2518-2529. https://doi.org/10.1093/jac/dkq347.

Wang, W., Tian, D., Hu, D., Chen, W., Zhou, Y., Jiang, X., 2023. Different regulatory mechanisms of the capsule in hypervirulent *Klebsiella pneumonia:* "direct" wcaJ variation vs. "indirect" rmpA regulation. Frontiers in Cellular and Infection Microbiology 13, 1108818. https://doi.org/10.3389/fcimb.2023.1108818.

Wareth, G., Linde, J., Hammer, P., Nguyen, N.H., Nguyen, T.N.M., Splettstoesser, W.D., Makarewicz, O., Neubauer, H., Sprague, L.D., Pletz, M.W., 2020. Phenotypic and WGS-derived antimicrobial resistance profiles of clinical and non-clinical *Acinetobacter baumannii* isolates from Germany and Vietnam. International Journal of Antimicrobial Agents 56, 106127. https://doi.org/10.1016/j.ijantimicag.2020.106127.

Wareth, G., Melzer, F., Neubauer, H., 2017. In *Brucella*: Selective pressure may turn some genes on instead of default off position. Medical Hypotheses 103, 29-31. https://doi.org/10.1016/j.mehy.2017.04.006.

Wareth, G., Sprague, L.D., Neubauer, H., Pletz, M.W., 2021. *Klebsiella pneumoniae* in Germany: An overview on spatiotemporal distribution and resistance development in humans. German Journal of Microbiology 1, 16-25. https://doi.org/10.51585/gjm.2021.0004.

Windham, S., Kollef, M.H., 2022. How to use new antibiotics in the therapy of serious multidrug resistant Gram-negative infections? Current Opinion in Infectious Diseases 35, 561-567. https://doi.org/10.1097/qco.0000000000000858.

Wood, D.E., Lu, J., Langmead, B., 2019. Improved metagenomic analysis with Kraken 2. Genome Biology 20, 257. https://doi.org/10.1186/s13059-019-1891-0.

Xu, L., Sun, X., Ma, X., 2017. Systematic review and meta-analysis of mortality of patients infected with carbapenem-resistant *Klebsiella pneumoniae*. Annals of clinical microbiology and antimicrobials 16, 18. https://doi.org/10.1186/s12941-017-0191-3.

Yen, N.T.P., Nhung, N.T., Phu, D.H., Dung, N.T.T., Van, N.T.B., Kiet, B.T., Hien, V.B., Larsson, M., Olson, L., Campbell, J., Quynh, N.P.N., Duy, P.T., Carrique-Mas, J., 2022. Prevalence of carbapenem resistance and its potential association with antimicrobial use in humans and animals in rural communities in Vietnam. JAC Antimicrobial Resistance 4, dlac038. https://doi.org/10.1093/jacamr/dlac038.

Yuan-Chuan, C. 2020. Introductory Chapter: Gene Expression and Phenotypic Traits, In Yuan-Chuan, C., Shiu-Jau, C. (Eds.) Gene Expression and Phenotypic Traits. IntechOpen, Rijeka, Ch. 1. http://dx.doi.org/10.5772/intechopen.89863.

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., Aarestrup, F.M., Larsen, M.V., 2012. Identification of acquired antimicrobial resistance genes. The Journal of Antimicrobial Chemotherapy 67, 2640-2644. https://doi.org/10.1093/jac/dks261.

Zhang, R., Lin, D., Chan, E.W., Gu, D., Chen, G.X., Chen, S., 2016. Emergence of carbapenem-resistant serotype K1 hypervirulent *Klebsiella pneumoniae* Strains in China. Antimicrobial Agents and Chemotherapy60, 709-711. https://doi.org/10.1128/aac.02173-15