Whole Genome Analysis of the Extensively Drug Resistant Clinical *Klebsiella Pneumonia* Isolates from the Military Hospital in Vietnam

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Abstract

Background

The over using of antibiotics in hospitals, communities and agriculture has raised drug resistance in Vietnam. Recently, *Klebsiella pneumoniae* (*K.pneumoniae*) emerged as the main agent causing hospital infections because of the alarming resistance to carbapenem and high mortality. The purpose of this study was to identify the antibiotic resistance pattern, gene carbapenemase expression and mutation that related to drug resistance mechanisms of *K.pneumoniae* isolated from clinical samples in the Military Hospital 103 in Vietnam.

Methods

Seventeen extensively drug resistant (XDR) strains were completed whole genome sequence, analyzed by bioinformatics, and confirmed using PCR and Sanger sequence methods.

Results

K.pneumoniae showed resistance to almost fifteen antibiotics with very high rates of over 70%, only sensitive to colistin 100%. There is a correlation between carbapenem resistance and XDR status in the *K.pneumoniae* group. Analysis Multilocus sequence typing (MLST) reported the total of 9/17 strains belong to ST15, after that ST11 (4/17), ST656 (2/17), ST 16 (1/17) and ST395 (1/17). Most of the *K.pneumoniae* isolates harbored the widely distributed ESBL genes, e.g. *NDM*, *SHV*, *OXA*, *KPC*, *TEM* and *CTX-M*, in which, the most prevalent carbapenemase genes were *NDM-1*, *NDM-4*, *OXA-48*, *OXA-181* and *KPC-2*. Especially, two strains demonstrated resistance to a wide range of antibiotics and showed the novel drug resistance gene mutation, namely Pro187Ala in *NDM-4* gene and Gly25Ser in *KPC-2* gene of MH16-335M and MH17-011M, respectively.

Conclusion

Our study adds new and significant DNA sequence data on *K.pneumoniae* strains and demonstrates the value of whole-genome sequencing in characterizing multidrug resistance in clinical isolates.

Introduction

In the near decades, the problem of concern has been the rapid increase of strains of bacteria resistant to many new antibiotics, especially resistant strains of Gram-negative bacteria such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* [1]. Currently, antimicrobial resistance of Gram-negative bacilli is a persistent issue [2], and becoming more and more difficult to treat [3]. Over time, resistance develops with all antibiotics and spreads. The increasing clinical incidence of antibiotic resistant bacteria is a serious issue for global health care. Understanding the characterization of antibiotic resistance determinants at the genomic level plays a critical role in potentially controlling the spread of multidrug resistant pathogens [4].

The rapid dissemination of multiple drug resistance (MDR) and XDR or pan drug resistant (PDR) *K.pneumoniae* has recently recognized by the CDC as an urgent public health threat, thus requiring immediate and aggressive actions [5]. In immunocompromised individuals, neonates and the elderly, *K.pneumoniae* can cause severe hospital or community acquired infections (Brisse et al., 2009). The current antimicrobial resistance and susceptibility of *K.pneumoniae* bacteria have been observed clinically [6]. Some clinical *K.pneumoniae* isolates are resistant to all available antibiotics [7]. The acquisition of antibiotic resistance genes and intrinsic resistance of bacteria has consequently limited treatment options for infections caused by *K.pneumoniae*. Currently, *K.pneumoniae* strains producing Extended Spectrum Beta-Lactamases (ESBLs) and carbapenemases have spread globally [8]. The frequent outbreak of nosocomial infections due to extended spectrum β -lactamase of *K.pneumoniae* that is attributed to multiple drug resistance mechanisms [9]. The mechanisms involved when β -lactamase hydrolyzes β -lactam antibiotics or inactivates β -lactams have been investigated [10]. However, no precise molecular biological mechanisms underlying the antimicrobial drug resistance of *K.pneumoniae* have been reported [11]. In the previous several studies indicated that antibiotic resistance genes originate in microorganisms and subsequently integrate into the genome of other pathogens through transduction and/or transformation to produce a multitude of different antibiotic resistance strains that are especially dangerous for the course of treatment for patients [12–14]. Antimicrobial resistance genes in *K.pneumoniae* is also associated with high rates of morbidity and mortality in clinical patients [15]. Therefore, understanding the mechanisms involved in the antibiotic resistance of *K.pneumoniae* are highly required to warrant the development of novel antibiotics or alternative treatment strategies to decrease mortality of patients infected with *K.pneumoniae*.

Carbapenem is the widest antimicrobial spectrum and stable activity to against bacteria that produce AmpC β -lactamase and ESBL antibiotic enzymes, therefore, this antibiotic is considered last treatment options in the treatment of infections caused by multiple antibiotics resistant gram-negative bacteria [16]. However, the increasing of carbapenem resistant (CRE) isolates parallelly with their global distribution highlights the rapid dissemination of MDR genes to other highly virulent nosocomial pathogens [17]. Carbapenemase genes are frequently encoded on plasmids or other mobile genetic elements that can potentially be carrying additional resistance genes against other classes of antibiotics [18]. Besides, the major contributing factor to the rapid global spread of carbapenem resistance is transmissible plasmids that carry carbapenemase genes [19, 20], namely, *K.pneumonia* carbapenemase (KPC), New Delhi metallo- β -lactamase (NDM), and oxacillinase (OXA) type carbapenemases [21]. The other genetic mechanisms of carbapenem resistance include efflux pumps, altered function or expression of porins and penicillin-binding proteins (PBPs) [22].

Whole-genome sequencing (WGS) to be established as a viable option to antimicrobial susceptibility characterization of the clinical isolates, the association between the resistance genotype and phenotype, and the characterization of carbapenem resistance factors and to assess the risk for horizontal transfer and dissemination of carbapenemase genes. This is especially important to many carbapenem-resistant pathogens which often exhibit complex phenotypic profiles with resistance to multiple classes of antibiotics. In addition, the use of such high throughput technologies to study pathogens has brought a breakthrough in surveillance, monitoring and diagnostics in public health. In this study, we used WGS in understanding antimicrobial resistance, identifying resistance determinants and predicting genotype resistance in *K.pneumoniae* isolated from patients who were admitted to the Military Hospital 103 (MH103), a military central hospital in Northern Vietnam.

Material And Methods

K.pneumoniae sample collection

The seventeen *K.pneumoniae* strains analysed in the present study were isolated between 2015 and 2017 in the Military Hospital 103, and were selected due to their extensively drug resistant phenotypes. The strains originated from inpatients in hospital, who were sampled due to signs of infection. The identification of *K.pneumoniae* isolate was done by analyzing colony morphology in special culture media, microscopic examination, performing biochemical testing, and Vitek 2 system (bioMérieux, Marcy l'Étoile, France).

Resistant antibiotic phenotype identification of *K.pneumoniae* strains

Testing of antibiotics susceptibility and carbapenemase producing capacity used the Vitek 2 compact system and E-test method. Antimicrobial susceptibilities were performed for 17 *K.pneumoniae* isolates using the Vitek 2 system and the AST-N204 card (bioMérieux, Marcy l'Étoile, France). Values were interpreted according to breakpoint table for interpretation of MIC values and zone diameters (CLSI, 2020). Colistin susceptibility assay was performed according to recommendations of joint CLSI-EUCAST guidelines:

http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/Recommendations_for_MIC_determination_of_colistin_March_2016.pdf (Chandler C. Roe, 2019). The MIC of the antibiotics carbapenem was measured for all isolates using Etest (bioMérieux) according to the manufacturer's instructions. The results were used to classify the isolates as either susceptible, intermediate or resistant to the tested antibiotics, based on CLSI (Clinical and Laboratory Standard Institute) breakpoints [23]. Antibiotics used as antibiotics include: Ampicillin (AM), Ampicillin/ Clavulanic acid (AMC), Piperacillin/tazobactam (TZP), Cefotaxime (CTX), Ceftazidime (CAZ), Cefepime (FEP), Amikacin (AN), Gentamicin (GM), Ciprofloxacin (CIP), Norfloxacin (NOR), Fosfomycin (FOS), Nitrofurantoin (NIT), Sulfamethoxazole/Trimethoprim (SXT), Ertapenem (ETP), Imipenem (IPM), Meropenem (MEM), and Doripenem (DOR).

DNA extraction and species identification of *K.pneumoniae* strains

Total bacterial DNA was extracted from the bacteria that enriched on blood agar medium and incubating overnight. The DNA was extracted using a GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific) according to the manufacturer guideline. The quality and quantity of DNA were assessed using NanoDrop ND 1000 spectrophotometer, Qubit 2.0 Fluorometer (Thermo Fisher Scientific), Agilent 2100 Bioanalyzer system (Agilent), visualization on an agarose gel 1% and yield concentration reached 200 ng / μ L before sending DNA for sequencing. For the confirm of WGS data, DNA samples, primers of gene targets were prepared for PCR according to the protocol description. The *16S rRNA* gene of seventeen isolates was sequenced in the ABI3500 system (Applied Biosystem). The isolate sequences in this study, and 23 published reference sequences including twenty two *Enterobacteriaceae* strains and one out group strain were aligned using MUSCLE algorithms. The maximum likelihood tree with T92 + G substitution model and bootstrap 1000 were built to determine the genetic relationship. All analysis processes were performed using MEGA6 software [24].

Whole genome sequencing setup

All isolates were index tagged and sequenced at the National Institute of Infectious Diseases (Tokyo, Japan), using the HiSeq4000 system (Illumina) with the High Output Reagent Kit (300 cycles) and the MinION sequencer (Oxford Nanopore Technologies) with the R9.4.1 flowcell. The Nextera XT DNA Library Preparation Kit (Illumina) was used for preparing Illumina sequencing (150 bp paired-end, insert size of 500–900 bp), and the library for MinION sequencing was prepared using the Rapid Sequencing Kit (SQK-RAD004) according to the manufacturer recommendations. Basecalling for MinION reads was carried out by Guppy v.3.6.1.

Genotype identification and annotation

The raw HiSeq Illumina pair-end (PE) reads were trimmed of the adaptor sequences using BBDuk program from the BBTools suite (<https://jgi.doe.gov/data-and-tools/bbtools/>). The trimmed reads were assembled using SPAdes de novo assembler (<http://cab.spbu.ru/software/spades/>). Average Nucleotide Identity (ANI) was applied using the FastANI program (<https://github.com/ParBLISS/FastANI>) on the resulting scaffolds from SPAdes, in order to double-check the species against the *K.pneumoniae* complete assembled reference genomes downloaded from NCBI. Genome sequences were annotated using the DFAST server (<https://dfast.nig.ac.jp>). Multi locus sequence typing (MLST) and the sample subtype (ST) were predicted according to Pasteur's Klebsiella sequence typing method (<https://bigsd.b.pasteur.fr/klebsiella/klebsiella.html>). Antimicrobial resistance genes were detected using ResFinder v.4.0, respectively, on the CGE server (<http://www.genomicepidemiology.org>). Linear comparison of sequence alignment was performed using BLAST and visualized by Easyfig v.2.2.5 (<http://mjsull.github.io/Easyfig/>). Mapping Illumina reads and calling sequence variants were performed using CLC Genomics Workbench. Finally, reference mapping and BLAST searches of the contigs have been performed with Geneious Prime 2019.2.1 (<https://www.geneious.com>).

Confirmation and mutation analysis of antibiotic resistance genes in *K.pneumoniae* isolates using PCR and Sanger sequencing

The distribution of genes associated with antimicrobial resistance was identified for all *K.pneumoniae* genomes. Acquired genes associated with antimicrobial resistance were identified using the ResFinder database (Kleinheinz et al., 2014). The ResFinder database included genes that may confer resistance to the following antibiotics: aminoglycosides, beta-lactams, colistin, fluoroquinolone, fosfomycin, fusidic acid, glycopeptide, macrolide, lincosamide, and streptogramin B (MLS), nitroimidazole, oxazolidinone, phenicol, rifampicin, sulphonamide, tetracycline, and trimethoprim. The presence of carbapenem resistance related genes such as *NDM-1*, *NDM-4*, *KPC-2*, *OXA-181*, and *OXA-48* in 17 *K.pneumoniae* isolates was examined by PCR with specific primers (Table 1). Mutations within the *NDM-1*, *NDM-4*, *KPC-2*, *OXA-181*, and *OXA-48* genes that are known to confer resistance to carbapenem were also investigated using Sanger sequencing.

Table 1 Primer sets used for screening gene targets				
Gene name	Sequence (5' to 3')		Size (bp)	Reference
NDM	Fwd	CACCTCATGTTTGAATTCGCC	984	[25]
	Rev	CTCTGTCACATCGAAATCGC		
OXA	Fwd	ATGCGTGTATTAGCCTTATCG	888	[26]
	Rev	AACTACAAGCGCATCGAGCA		
KPC	Fwd	TGTCACGTATCGCCGTC	1011	[27]
	Rev	CTCAGTGCTCTACAGAAAACC		
16S	27F	AGAGTTTGATCCTGGCTCAG	1465	[28]
rRNA	1492R	TACGGYTACCTGTTACGACTT		

Results

Characteristics of clinical samples

From January 2015 to December 2017, *K.pneumoniae* isolates were randomly examined from over 1000 total clinical bacterial isolates at the Military Hospital 103, Vietnam. *K.pneumoniae* isolates were isolated from 14 male (84.4%) and 03 female (17.6%) patients. Most of *K.pneumoniae* were isolated from patients aged from 23 to 92 years old. Besides, *K.pneumoniae* samples were mostly isolated from Intensive Care Unit (ICU) (71.4%) and non-ICU (9.6%) (Table 2)

Table 2
Sociodemographic characteristics associated with 17 *K. pneumoniae* bacterial isolates from patients who were admitted to the military hospital 103

ID	Collection year	Age (years)	Gender	Location	Source of sample	Ward
MH15-208H	2015	76	M	inx	sputum	Ort
MH15-258M	2015	92	M	ICU	blood	ICU
MH15-289M	2015	59	M	inx	blood	Nep
MH16-335M	2016	23	F	ICU	blood	ICU
MH16-390M	2016	63	M	inx	blood	Abd
MH16-398D	2016	88	M	ICU	bili	ICU
MH16-444M	2016	32	M	ICU	blood	ICU
MH16-519D	2016	46	M	ICU	sputum	ICU
MH17-536D	2017	47	M	ICU	sputum	ICU
MH17-004D	2017	65	F	ICU	sputum	ICU
MH17-007D	2017	49	M	inx	blood	inf
MH17-010D	2017	75	M	inx	urine	inf
MH17-011M	2017	57	M	inx	urine	inf
MH17-016D	2017	67	M	ICU	wound	ICU
MH17-017D	2017	54	F	ICU	sputum	ICU
MH17-553M	2017	56	M	ICU	sputum	ICU
MH17-556D	2017	63	M	inx	sputum	out

M: Male; F: Female; Inx: Inpatient (non-ICU); ICU: Intensive care unit; Ort: orthopedic; Nep: nephrology; Abd: abdominal surgery; Inf: infection department; Out: outpatient department;

Based on the genomes from the *K.pneumoniae* strains published on the ANI (Average Nucleotide Identity) data, a 16S rRNA-based subspecies analysis was conducted to determine the genetic relationships of the strains using in research. The Fig. 1 shows phylogenetic species of all *K.pneumoniae* isolates closely related in a group of *K.pneumoniae* strains ATCC_13883,11296, 13884 (77–97 bootstrap values), and belong to the family Enterobacteriaceae. Thus, all strains of *K.pneumoniae* isolated at the hospital have highly genetically similarity level with *K.pneumoniae* ATCC strains.

Antibiotic sensitivity and MIC of strains studied

Antimicrobial susceptibilities were performed for samples by the Vitek 2 system and the AST-GN card, E-test, and Broth microdilution (BMD) methods. The isolates were screened against with 18 antibiotics. The results showed that all *K.pneumoniae* isolates were still susceptible to colistin (100%); highly resistant to ampicillin, sulfamethoxazole/trimethoprim, and ertapenem (82.4–94.1%); and completely resistant (100%) to ampicillin, piperacillin/tazobactam, cefotaxime, certapenem, cefepime, ciprofloxacin, and norfloxacin.

In particular, all 17 isolates in this study displayed extensively drug resistance. Besides, a study was designed to determine in vitro efficacy of Modified Hodge test (MHT) for detection of carbapenemase production in *K.pneumoniae* strains as guide in A Amjad, I Mirza, S Abbasi, U Farwa, N Malik and F Zia [29]. Out of a total 17 isolates which were showing intermediate or susceptible zone, i.e. 6 mm – 23 mm for meronem, 14/17 (82.4%) were positive for carbapenemase production by Modified Hodge test. In addition, the strains used in the study all had similar MICs with imipenem, meropenem and ertapenem and all were very high compared to the CLSI recommended standard, up to 32 µg / mL (Table 3).

Table 3
Phenotypic antimicrobial resistance pattern of the 17 *K. pneumoniae* isolates

ID (MIC/ CLSI)	VITEK																	Colistin COL (BMD)
	AM	AMC	TZP	CTX	CAZ	FEP	AN	GM	CIP	NOR	FOS	NIT	SXT	ETP	IMP	MEM	DOR	
MH15-208H	> 16	>16	>64	>32	>32	> 32	>32	>8	>2	>8	> 128	> 256	160	>4	>32	>32	>32	<0.25
	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S
MH15-258M	> 16	>16	>64	>32	>32	16	>32	>8	>2	>8	64	256	40	>4	2	6	4	<0.25
	R	R	R	R	R	R	R	R	R	R	S	R	S	R	I	R	R	S
MH15-289M	> 16	16	>64	>32	>16	8	>32	>8	>2	>8	<=16	32	> 160	< 0.5	0.19	0.032	0.032	<0.25
	R	R	R	R	R	R	R	R	R	R	S	S	R	S	S	S	S	S
MH16-335M	> 16	>16	>64	>32	>32	> 32	16	>8	>2	>8	32	128	> 160	>4	16	16	12	<0.25
	R	R	R	R	R	R	S	R	R	R	S	S	R	R	R	R	R	S
MH16-390M	> 16	>16	>64	>32	>16	> 32	<=2	<=1	>2	>8	> 128	128	> 160	>4	8	8	>32	<0.25
	R	R	R	R	R	R	S	S	R	R	R	S	R	R	R	R	R	S
MH16-398D	> 16	>16	>64	>32	>32	> 32	>32	>8	>2	>8	> 128	> 256	40	>4	2	8	8	<0.25
	R	R	R	R	R	R	R	R	R	R	R	R	S	R	I	R	R	S
MH16-444M	> 16	>16	>64	>32	>32	16	<=2	>8	>2	>8	> 128	64	> 160	>4	4	6	1.5	<0.25
	R	R	R	R	R	R	S	R	R	R	R	S	R	R	R	R	I	S
MH16-519D	> 16	16	32	>32	>32	4	<=2	>8	>2	>8	> 128	128	> 160	< 0.5	4	4	4	<0.25
	R	R	R	R	R	R	S	R	R	R	R	S	R	S	R	R	R	S
MH17-536D	> 16	16	>64	4	>32	> 32	<=2	<=1	>2	>8	> 128	128	> 160	>4	8	3	2	<0.25
	R	R	R	R	R	R	S	S	R	R	R	S	R	R	R	I	I	S
MH17-004D	> 16	>16	>64	>32	>32	> 32	<=2	>8	>2	>8	> 128	256	> 160	>4	2	4	3	<0.25
	R	R	R	R	R	R	S	R	R	R	R	R	R	R	I	R	I	S
MH17-007D	> 16	4	>64	>32	>32	> 32	<=2	<=1	>2	>8	<=16	64	> 160	< 0.5	0.125	0.047	0.032	<0.25
	R	I	R	R	R	R	S	S	R	R	S	S	R	S	S	S	S	S
MH17-010D	> 16	>16	>64	>32	>32	> 32	16	<=1	>2	>8	<=16	> 256	<=20	>4	>32	>32	>32	<0.25
	R	R	R	R	R	R	S	S	R	R	S	R	S	R	R	R	R	S
MH17-011M	> 16	>16	>64	>32	>32	> 32	16	>8	>2	>8	64	64	> 160	>4	>32	>32	>32	<0.25
	R	R	R	R	R	R	S	R	R	R	S	S	R	R	R	R	R	S
MH17-016D	> 16	>16	>64	>32	>16	> 32	<=2	<=1	>2	>8	<=16	> 256	> 160	>4	2	12	6	<0.25
	R	R	R	R	R	R	S	S	R	R	S	R	R	R	I	R	R	S
MH17-017D	> 16	>16	>64	>32	>32	> 32	16	>8	>2	>8	<=16	128	> 160	>4	2	4	4	<0.25
	R	R	R	R	R	R	S	R	R	R	S	S	R	R	I	R	R	S
MH17-553M	> 16	>16	>64	>32	>32	> 32	16	>8	>2	>8	> 128	64	> 160	>4	6	2	2	<0.25

ID (MIC/ CLSI)	VITEK																	Colistin COL (BMD)
	AM	AMC	TZP	CTX	CAZ	FEP	AN	GM	CIP	NOR	FOS	NIT	SXT	ETP	IMP	MEM	DOR	
	R	R	R	R	R	R	S	R	R	R	R	S	R	R	R	I	I	S
MH17-556D	> 16	> 16	> 64	> 32	> 32	> 32	16	> 8	> 2	> 8	<=16	128	> 160	> 4	8	4	4	< 0.25
	R	R	R	R	R	R	S	R	R	R	S	S	R	R	R	R	R	S
R (%)	100	94.1	100	100	100	100	23.5	70.6	100	100	47.1	35.3	82.4	82.4	64.7	76.4	64.7	0
S (%)	0	0	0	0	0	0	76.5	29.4	0	0	52.9	64.7	17.6	17.6	11.8	11.8	11.8	100
I (%)	0	5.9	0	0	0	0	0	0	0	0	0	0	0	0	23.5	11.8	23.5	0

AM: Ampicillin, AMC: Ampicillin/ Clavulanic acid, TZP: Piperacillin/tazobactam, CTX: Cefotaxime, CAZ: Ceftazidime, FEP: Cefepime, AN: Amikacin, GM: Gentamicin, CIP: Ciprofloxacin, NOR: Norfloxacin, FOS: Fosfomycin, NIT: Nitrofurantoin, SXT: Sulfamethoxazole/Trimethoprim, ETP: Ertapenem, IPM: Imipenem, MEM: Meropenem, DOR: Doripenem,

MIC: Minimum Inhibitory Concentration,CLSI: Clinical and Laboratory Standard Institute, BMD: Broth microdilution, R – Resistant, S – Susceptible, I – Susceptible according to CLSI guidelines

Assembling and annotating genome sequence

The cleaned and paired reading datasets were used to assemble the de novo genome using the CLC Genomics Workbench v.9.0 (Qiagen), resulting in *K.pneumoniae* genome sizes were 5,533,931 bp to 5,907,064 bp in length; N50 is from 44,277 to 198,000 bp and the number of contig contents from 132 (MH17-556D) to 377 (MH15-258M). *In silico* MLST typing was performed on all assemblies using the previously described Pasteur system [30]. All of *K.pneumoniae* isolates analyzed, nine were assigned to ST101, four were assigned to ST15, two were assigned to ST656, and one sample such as MH17-010D or MH15-208H was assigned to ST16 and ST395, respectively (Table 4).

Table 4 Genome characteristics of the sequenced <i>K.pneumoniae</i> isolates in this study						
ID	Genome size	Contig	N50	Reads	Depth	MLST
	(bp)					
MH15-208H	5,703,625	139	198000	1234604045	216.46	ST395
MH15-258M	5,868,091	377	119033	2605895333	444.08	ST11
MH15-289M	5,771,413	159	112598	916431432	158.79	ST11
MH16-335M	5,840,408	274	78685	818667297	140.17	ST15
MH16-390M	5,638,714	219	64647	547610579	97.116	ST656
MH16-398D	5,640,361	263	97967	492303949	87.282	ST11
MH16-444M	5,640,233	288	70304	692240562	122.73	ST15
MH16-519D	5,682,307	287	44277	447151755	78.692	ST15
MH17-536D	5,652,240	292	50450	422652360	74.776	ST15
MH17-004D	5,694,352	280	86536	616909469	108.34	ST11
MH17-007D	5,637,531	149	152300	739237554	131.13	ST656
MH17-010D	5,608,215	160	152169	1468353285	261.82	ST16
MH17-011M	5,652,498	202	106211	1029225412	182.08	ST15
MH17-016D	5,907,064	233	105352	1591376564	269.4	ST15
MH17-017D	5,847,449	250	104402	1187311434	203.05	ST15
MH17-553M	5,673,369	177	105168	783978869	138.19	ST15
MH17-556D	5,533,931	132	104363	699158060	126.34	ST15

Approximate genome size in base pairs; Contig: a set of overlapping DNA segments that together represent a consensus region of DNA; N50 is the shortest contig length needed to cover 50% of the genome; ST: Multilocus sequence typing (MLST) sequence type.

In silico antimicrobial resistance genes profiling

From the analysis of genomic annotation using the DFAST server (<https://dfast.nig.ac.jp>), antimicrobial resistance genes and function were detected using ResFinder v.4.0 and Patric PlasmidFinder v.2.1, respectively (<http://www.genomicepidemiology.org>). Genes responsible for conferring aminoglycoside resistance, beta-lactam resistance, fluoroquinolone, aminocyclitol, folate pathway antagonist, macrolide, rifamycin, and tetracycline resistance were presented across all genomes of *K.pneumoniae* (Table 5).

Table 5
Distribution of multiple carbapenem resistance genes identified among the isolates

ID	NDM – 1	NDM 4	KPC – 2	OXA 181	OXA 48	Total
MH15-208H	1	0	0	0	0	1
MH15-258M	1	0	0	0	0	1
MH15-289M	0	0	0	0	0	0
MH16-335M	0	1	0	0	0	1
MH16-390M	1	0	0	0	0	1
MH16-398D	1	0	0	0	0	1
MH16-444M	0	0	1	0	0	1
MH16-519D	0	0	1	0	0	1
MH17-536D	0	0	1	0	0	1
MH17-004D	0	0	1	0	0	1
MH17-007D	0	0	1	0	0	1
MH17-010D	0	1	0	1	0	2
MH17-011M	0	0	1	0	0	1
MH17-016D	0	1	0	0	1	2
MH17-017D	0	1	0	0	0	1
MH17-553M	0	0	1	0	0	1
MH17-556D	0	1	0	0	0	1

1: gene expression, 0: gene no expression
Antimicrobial resistance gene profile of seventeen resistant *K.pneumoniae* isolates was reported in Supplementary Table 1. Briefly, fluoroquinolone resistance genes (*oqx*A, *oqx*B, and *aac*(6')-Ib-cr) were identified within every *K.pneumoniae* genome. Genes resistance for aminoglycoside and fosfomycin resistance were identified in 100% of *K.pneumoniae* genomes as *aac*(6')-Ib-cr and *fos*A, respectively. Additionally, other aminoglycoside resistance genes were shown such as *rmt*B, *aac*(6')-Ib/Ib3, *aac*(3)-IIa/IIId, *aad*A, *aph*(6)-Id, and *aph*(3'')-Ib. A total of 8 different beta-lactam genes was shown in isolates (i.e., *CTX-M*, *SHV*, *OXA*, *KPC*, *NDM*, *TEM*, *DHA*, and *LAP*).

Aminocyclitol resistance gene were mainly *aad*A. About 35.3–76.5% isolates carried genes responsible for folate pathway antagonist (*sul* and *dfr*A). Phenicol resistance genes (*cat*A, *cat*B and *flor*) were shown in 6–47% of the *K.pneumoniae* genomes, and 52.9% of isolates carried genes responsible for macrolide (*mph*A). Rifampicin resistant genes (*arr*-2 and *arr*-3) were present in 5.9–47.1% of genomes, and other tetracycline resistance genes (*ram*R, *tet*A, *tet*D and *acr*R). Especially, a list of fluoroquinolone, carbapenem, and cephalosporins resistance genes such as *aac*(6')-Ib-cr, *ompK37*, *ompK376*, and *acr*R was found in 88.2–100% of the *K.pneumoniae* genomes.

Analysis of molecular characterization of the relevant carbapenem resistance genes using PCR and Sanger sequencing

There is little knowledge about carbapenemase-producing *K.pneumoniae* in Vietnam. The confirm characterization of presumptive carbapenem resistant genes from WGS analysis were used polymerase chain reaction (PCR) and Sanger sequencing. Out of 17 *K.pneumoniae* that showed resistance against the test antibiotics, 94.1% isolates were confirmed to harbour carbapenem resistance genes and the result is shown in Table 5. Five tested target genes (*NDM-1* and *NDM-4*, *KPC-2*, *OXA-48* and *OXA-181*) were expressed in most of *K.pneumoniae* isolates, whereas only one strain (MH15-289M) was not expressed any tested gene. Two isolates were carried two carbapenem resistance genes such as MH17-010D (*NDM-4* and *OXA-181*) and MH17-016D (*NDM-4* and *OXA-48*).

The mutations in carbapenem resistance genes (*NDM-1*, *NDM-4*, *KPC-2*, *OXA-181*, and *OXA-48*) were screened by DNA sequencing. Results of analysis and comparison of the sequence of the *KPC-2* gene encoding for KPC-2 enzyme of *K.pneumoniae* isolates with the reference *K.pneumoniae* genome data (NG 049253.1) using bioinformatics software, indicated the missens change in Gly25er (acid amin 25 Glycine changed to a Serine) in MH17-011M (Fig. 2). And the mutation in the location of *NDM-4* gene was Pro187Ala (amino acid 187 Proline changed to an Alanine) in MH16-335M (Fig. 3). Other *OXA* genes were not shown any mutation in *K.pneumoniae* isolate sequences (Fig. 4).

Discussion

The tracking resistance patterns, changing the prescribing habits, and increasing the infection control are the best way in reducing the development of bacterium antibiotic resistance, improving and saving in treatment to patients and health care facilities. The nosocomial infections by *K.pneumoniae* are still prevalent, and may be more dangerous due to the rapid development and spread of antimicrobial resistance in hospital [31]. In this study, most of the XDR *K.pneumoniae* isolates were collected from male patients. This result was similarly with a survey data from health care centers in Nigeria, here *K.pneumoniae* infection was higher in males than females [32]. Akter *et al.* also reported that male patients had a higher risk to get *Klebsiella* infection than females J Akter, AMMA Chowdhury and MAI Forkan [33]. There may be a association in poor lifestyle choices, smoking and alcoholism between male and female [32]. In addition, most of *K.pneumoniae* in this study were isolated from patients aged 23 to 92 years old. In the previous studies revealed a greater number of *K.pneumoniae* isolates were obtained from patients aged among 40 to 65 years or over 70 years old [34, 35]. The differences in age distribution of patients may be related to the response of the immune system, such as under 40 years have stronger immune systems, and more pressure to fight to *K.pneumoniae* [36]. Besides, an increasing age leads to increase the comorbid illness cause of a higher risk of *K.pneumoniae* infection [37]. These results also revealed that *K.pneumoniae* isolates were mainly isolated from ICU and respiratory specimens (namely is sputum samples). Ashurst and Dawson [38] emphasized that *K.pneumoniae* typically colonizes human mucosal surfaces of the oropharynx. For this reason, *K.pneumoniae* is considered to be the most common cause of hospital acquired pneumonia in the Vietnam [39]. The finding is in line to a study by Wang *et al.*, which reported that the respiratory tract was the main infection site of *K.pneumoniae* in China [40].

Antimicrobial resistance bacteria are commonly associated with nosocomial infections cause of the overuse of antibiotics, and without monitoring or control [41]. In this study, most of *K.pneumoniae* was resisted to various antibiotics, fully resistant to ampicillin, piperacillin/tazobactam, cefotaxime, ceftazidime, cefepime, ciprofloxacin, and norfloxacin, whereas colistin, amikacin, nitrofurantoin, and fosfomycin were being the most effective for *K.pneumoniae*. The finding was higher than other results, Cepas *et al.* reported that 40% of *K.pneumoniae* strains were resisted to ciprofloxacin and amoxicillin-clavulanic acid [42]. Manjula *et al.* showed that 90.2% of *K.pneumonia* isolates were MDR, and high resistance to penicillin, cephalosporin, fluoroquinolone, aminoglycoside, and sulfonamide [43]. These MDR microbes are causing a great challenge in controlling infections, thus it is very important to monitor and optimize antibiotic use through antibiotic management programs [44, 45].

In the resistant phenotype of *K.pneumoniae*, the XDR phenotype showed resistance to most antibiotics, especially strong resistance to carbapenem, evidencing that they hold a variety of resistance mechanisms. They are not only secreting the enzyme that destroys carbapenem but also showing the resistance mechanisms such as the porin channel closes and the pump expels antibiotics out. Previous studies have shown that antibiotic consumption leads to selective pressure increasing β -lactam resistance in the genus Enterobacteriaceae bacteria [46]. However, XDR may also a result of gene circulation in the bacterial population. Currently in Vietnam, research on carbapenem resistant genotype of *K.pneumoniae* has not been conducted much. In this study, whole genome sequencing of XDR *K.pneumoniae* strains, analysis and confirmation of carbapenem resistance genes, also the relationship between carbapenem resistance and multi-drug resistance of the strains of *K.pneumoniae* were performed. This will provide a clearer and more indepth look at about the XDR capacity of these strains. Currently, millions of MDR genes have been sequenced that includes mostly bla genes (*TEM*, *SHV*, *KPC*, *CTX-M*, *OXA*, *VIM*, *CMY*, *GIM*, and *SPM* etc), drug modifying genes like *catB*, *aadA*, *aacA*, *aph*, as well as *sul*, *arr3*, *dhfr*, *mcr-1* and *vanA* genes [47–49]. Antimicrobial resistance genes can be transferred to other bacterial species and thereafter can lead to the production of various enzymes in order to inactivate antimicrobial activities. The several members of Enterobacteriaceae group also can against carbapenems when combined mutations in chromosomal porin to prevent accumulation of β -lactam agents in the bacteria [50]. This study was also determined the antimicrobial susceptibility gene lists (Supplementary Table 1), the presence and mutation of five relevant carbapenem genes such as *KPC-2*, *NDM-1* and 4, and *OXA-48* and 181. The *K.pneumoniae* isolates had 93 difference resistance genotypes separating in 10 antibiotic groups (e.g., aminocyclitol, aminoglycoside, beta-lactam, fluoroquinolone, folate pathway antagonist, fosfomycin, macrolide, rifamycin, tetracycline, and under development groups). Among the resistance against carbapenems *K.pneumoniae* isolates, the results have shown a high percentage of harboring the *KPC-2* gene (41.2%), *NDM-4* gene (29.4%), *NDM-1* gene (23.5%), and a low percentage of both *OXA-48* and *OXA-181* genes (only 1/17 strain, accounting 5.9%). These findings were in accordance with the other reports that showed a low percentage of the *OXA-48* gene in *K.pneumoniae* isolates [51, 52], whereas a previous study in Romania was revealed that *OXA-48* was the most predominant genotype, followed by *NDM-1* and *KPC-2* in the carbapenem resistance *K.pneumoniae* clinical isolates [53]. There are a global epidemiology of the spread of carbapenem resistant *K.pneumoniae* strains with mainly carbapenemase genes as *KPC-2* in Bulgaria [54], *KPC-3* in Portugal [55], *OXA-48* in Spain [56] and Vietnam [57], *OXA-232* in China [58] and *NDM-1* in Nepal [59]. Hoang *et al.* also reported similar distributions of *NDM* and *KPC*-producing *Klebsiella* spp. in hospital and aquatic isolated in Vietnam [60].

In particularly, the mutations in *KPC-2* gene, a main gene is causing the carbapenem resistance of *K.pneumoniae*, has been identified. This mutation is located in position acid amin 25 Glycine changed to a Serine. Another mutation on *NDM-4* gene also indicated as amino acid 187 Proline changed to an Alanine. These mutations have not been mentioned in previous studies in carbapenem resistant *K.pneumoniae* strains. Mutations in DNA can have a significant impact on the resistance phenotype and hence the amino acid substitutions detected in two *K.pneumoniae* isolates may result in high levels of carbapenem resistance (Table 3). Although, the antibiotic resistance mechanism of these related genes has not been studied in this experiment, this helps researchers to localize and be more interested in these gene groups in other related studies. An interesting note in our study, of the two isolates containing mutations (MH16-335M and MH17-011M) were belongs to the multilocus sequence type ST15. The most frequent STs of carbapenem resistant *K.pneumoniae* are characterized by different strains that distributed geographically, namely, ST258 being predominant in Europe and USA, ST11 being most common in East Asia, whereas ST15 is a less frequently occurring strain but has been indicated in clinical cases within outbreaks worldwide [45]. In the specific outbreaks, ST15 isolates can be highly homogenic, a high variability in antibiotic susceptibility and potential horizontal gene acquisition (Björn Berglund, 2019). *K.pneumoniae* ST15 strains had few reports from Asia or Vietnam previously, however, near time, Jiansheng Huang *et al.* was the first description of nosocomial outbreaks caused by *K.pneumoniae* ST15 strains in China [61]. This may indicate the spread of ST15 strains between geographically close regions. These findings may contribute to understanding the existence and spread of resistant bacteria such as strain ST15. Although ST15 strains are low in carbapenem resistance and virulence, it is important to note the importance of careful assessment of phenotypic and genetic characteristics for increasing anti infection treatment.

Conclusion

In conclusion, our systematic study encountered predicting a resistance gene profile from whole genome data, the mutation in relating the carbapenemase genes and other alterations in clinical antibiotic resistance *K.pneumoniae* isolates. The identification of specific genomic variations of the drug resistant bacteria is important for the development of more effective pathogen control and treatment strategies. In the future, it is importance in continuing the description of the genetic mechanisms that associated resistant phenotype bacteria and their antibiotic resistant genotypes, namely, the discovery of novel genes, the altered role of intrinsic genes in multi drug resistance mechanism of bacteria.

Abbreviations

Whole-genome sequencing (WGS)

Intensive Care Unit (ICU)

Carbapenem resistant (CRE)

Extensively drug resistant (XDR)

Pan drug resistant (PDR)

Multiple drug resistance (MDR)

Sequence type (ST)

Multilocus sequence typing (MLST)

Declarations

Ethics approval and consent to participate

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of the medical faculty of the Military Hospital 103.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

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Figures

Figure 1

Phylogenetic tree was built using Maximum-Likelihood algorithm, bootstrap 1000. Vietnam: mean the seventeen multidrug-resistance *K.pneumoniae* isolated in Military Hospital 103.

Figure 2

KPC-2 protein sequence alignment for *K.pneumoniae* isolates with some known sequences.

Figure 3

NDM protein sequence alignment for *K.pneumoniae* isolates with some known sequences. The amino acid 154M>L: pMet154Leu (Methionine instead of Leucine) was identified as the protein coding for the NDM-4 gene.

Figure 4

OXA protein sequence alignment for *K.pneumoniae* isolates with some known sequences. The amino acid 104T>A: pThr104Ala (Threonine instead of Alanine), amino acid 110N>D: pAsn110Asp (Asparagin instead of Acid Aspactic), amino acid 168E>Q: pGlu168Gln (Acid Glutamic instead of Glutamine), and amino acid 171S>A: pSer171Ala (Serine instead of Alanine) were identified as the protein coding for the OXA-181 gene.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryTable1.doc](#)