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RESEARCH ARTICLE

NDM-1 and KPC-3 co-producing *Klebsiella pneumoniae* ST512 in bronchial secretion from a patient in an intensive care unit of a Greek Tertiary Care Hospital

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ABSTRACT

This study investigated a strain of *Klebsiella pneumoniae*, identified as GRTHES, which exhibited extensive antibiotic resistance. The strain was resistant to all beta-lactams, including combinations with newer agents such as meropenem/vaborbactam and imipenem/relebactam, as well as to aminoglycosides, fluoroquinolones, fosfomycin, trimethoprim-sulfamethoxazole and colistin. It remained susceptible to tigecycline. Whole-genome sequencing was performed by Ion Torrent platform on the *K. pneumoniae* strain. Genomic analysis revealed a genome length of 5,808,650 bp and a GC content of 56.9%. Advanced sequencing techniques and bioinformatic tools were used to assess resistance genes and plasmid replicons, highlighting the emergence of multidrug resistance and virulence traits. The strain carried $bla_{\text{NDM-1}}$ and $bla_{\text{KPC-3}}$ genes and was designated to KL107 O2afg type. Colistin resistance-associated *mgrB/pmrB* gene mutations were present, and the strain also harbored yersiniabactin-encoding *ybt* gene. Our findings provide insights into the genomic context of $bla_{\text{NDM-1}}$ and $bla_{\text{KPC-3}}$ carbapenemase-producing *K. pneumoniae* and emphasize the importance of continuous surveillance and novel therapeutic strategies to combat multidrug-resistant bacterial infections. It is the first time that an NDM-1 and KPC-3 co-producing strain of *K. pneumoniae* ST512 is identified in Greece. This study highlights the essential role of genomic surveillance as a proactive strategy to control the spread of carbapenemase-producing *K. pneumoniae* isolates, particularly when key antimicrobial resistance genes, such as $bla_{\text{NDM-1}}$ and $bla_{\text{KPC-3}}$, are plasmid-mediated. Detailed characterization of these isolates could reveal plasmid similarities that facilitate adaptation and transmission within and between hospitals. Although data on patient movements are limited, it is plausible that carbapenem-resistant isolate was selected to co-produce KPC and NDM through plasmid acquisition.

KEYWORDS

carbapenemase, antimicrobial resistance, whole-genome sequencing

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INTRODUCTION

The *Klebsiella pneumoniae* clones ST258 and its derivative ST512 pose significant concerns due to their widespread presence worldwide, driven by hospital-acquired transmission and the presence of carbapenem resistance genes, as revealed by genomic analyses [1]. In Greece,

ST512 is a prevalent clone, part of the CG258 clonal group that includes high-risk clones known for carbapenem resistance. Studies indicate that ST258 and ST512 are among the dominant sequence types in Greek hospitals, contributing notably to the dissemination of carbapenemase-producing *K. pneumoniae* [2]. A recent ECDC survey of Greek hospitals identified ST258/512, along with ST39 and ST323, as the most common carbapenem-resistant *K. pneumoniae* strains. ST512 is frequently associated with the $bla_{\text{KPC-2}}$ gene and has been implicated in hospital outbreaks worldwide, including in Italy, Greece, and the U.S. It is particularly associated with intensive care unit (ICU) settings where there is intensive antibiotic usage and high patient turnover, making it highly transmissible and persistent.

In addition, ST11 and ST15 clones producing NDM carbapenemase are endemic in Greek hospitals [3]. Unlike KPC enzymes, NDM carbapenemases are not inhibited by avibactam. Since the publication of the draft genome of an NDM-1 and KPC-2 co-producing *K. pneumoniae* strain from China in 2018 [4], *K. pneumoniae* strains carrying multiple carbapenemase genes, such as bla_{NDM} and bla_{KPC}, have spread globally. The coexistence of NDM and KPC carbapenemases with other antimicrobial resistance (AMR) mechanisms, including extended-spectrum β-lactamase (ESBL) or aminoglycoside-modifying enzymes, and virulence factors like the siderophore yersiniabactin, makes bloodstream infections (BSIs) caused by these strains difficult to treat. Unlike KPC-2 and KPC-3, NDM-1 and other metallo-β-lactamases (MBLs), such as IMP-1 or VIM-1, are not inhibited by avibactam, typically combined with ceftazidime.

This study presents a comprehensive genomic analysis of an ST512 strain of *K. pneumoniae*, isolated from the ICU of a Greek tertiary Hospital on August 26, 2023, focusing on antibiotic resistance, virulence factors, and plasmid content. Whole-genome sequencing (WGS) and bioinformatic analysis identified numerous resistance genes, including $bla_{\text{NDM-1}}$, $bla_{\text{KPC-3}}$, $bla_{\text{OXA-1}}$, and $bla_{\text{CTX-M-15}}$, contributing to extensive multidrug resistance (MDR), especially to beta-lactams and carbapenems. The strain harbors multiple plasmid types, such as IncFIA(HI1), IncFIB(K), IncFII(K), and IncX3, facilitating horizontal gene transfer. Additionally, point mutations like *gyrA S83I* confer resistance to fluoroquinolones, and *mgrB G37S, pmrB R256G* to colistin, respectively, further complicating the treatment options. The presence of virulence factors, including siderophore genes for iron acquisition and adhesion proteins, underscores this strain's pathogenic potential. Genome assembly statistics confirmed sequencing quality, providing a reliable basis for future research on resistance mechanisms and potential therapeutic interventions for infections caused by *K. pneumoniae*. This study marks the first report of *K. pneumoniae* ST512 co-producing NDM-1 and KPC-3 carbapenemases in Greece.

MATERIALS AND METHODS

Bronchial secretion of a male patient from the ICU of a Greek tertiary Hospital was investigated. Permission is not required as it was a routine sample. The bronchial secretion sample was processed in the microbiology department of the Hospital in Thessaloniki, Greece and cultured in routine culture media. *K. pneumoniae* strain was identified using Vitek-2 (Biomerieux, France). Identification and antimicrobial susceptibility testing were carried out by VITEK2 automated system (BioMerieux SA, Marcy L' Etoile, France) according to the manufacturer's instructions. Susceptibility to imipenem-relebactam and meropenem/vaborbactam was tested using minimum inhibitory concentration (MIC) test strips (Liofilchem, Roseto, Italy). The susceptibility results were interpreted according to the breakpoints of EUCAST (https://www.eucast.org/clinical_breakpoints accessed on 10-11-2024). Susceptibility to colistin was tested using cation adjusted broth microdilution method (Liofilchem, Roseto, Italy) [5]. Tigecycline susceptibility was evaluated using susceptibility breakpoints (susceptible ≤ 2 mg L^{-1}, intermediate 4 mg L^{-1}, resistant ≥ 8 mg L^{-1}), approved by the US Food and Drug Administration. Carbapenem resistance was defined as resistance to any of the carbapenems. The isolate was phenotypically tested for metallo-beta-lactamase (MBL) and *K. pneumoniae* carbapenemase (KPC) production using ethylenediaminetetraacetic acid (EDTA) and phenylboronic acid [6].

A multiplex lateral flow immunoassay (LFIA; NG-test CARBA 5; NG Biotech, Guipry-Messac, France) was used to detect common carbapenem-resistance genes, including bla_{KPC}, bla_{VIM}, bla_{NDM}, bla_{IMP}, and $bla_{\text{OXA-48-like}}$, in a single reaction [7]. Detection limits using purified enzymes for NDM, KPC, IMP, VIM, and OXA-48-like were 150 pg mL^{-1}, 600 pg mL^{-1}, 200 pg mL^{-1}, 300 pg mL^{-1}, and 300 pg mL^{-1}, respectively.

Polymerase chain rection (PCR) was performed for the common carbapenemase genes (Table 1). The primers used are shown in Table 1 (Biolegio, Nethrerlands).

The WGS was performed in a private laboratory in Greece. Libraries were prepared using the Ion Torrent technology and the Ion Chef Flow Diagram (Thermo Fisher Scientific, Waltham, MA, USA) [8]. The DNA libraries were sequenced using the 5SXLS system, and raw sequences were analyzed using the Ion Torrent Suite v.s1010 (Thermo Fisher Scientific). The online Galaxy Server tool and the Centre for Genomic Epidemiology database were then used. The quality of the reads was estimated using the FastQC tool (Galaxy version 0.75 + Galaxy 0); read quality was improved using a FASTQ Quality Trimmer with a sliding window (Galaxy version 1.1.5). Bacterial genome was assembled

Table 1. Primers used for PCR of *K. pneumoniae* strain GRTHES

NDM-F	5′ TGGCAGGACACTTCCTATC 3′
NDM-R	5′ AGATTGCCGAGCGAGCGACTTG 3′
VIM-F	5′ AGTGGTGAGTATCCGACA 3′
VIM-R	5′ ATGAAAGTGCGTGGAGAC 3′
KPC-F	5′ TCGCTAAACTCGAACAGG 3
KPC-2R	5′ TTAGTGCCCGTTGACGCCCAATCC 3′
OXA-48-F	5′ TTGGTGGCATCGATTATCGG 3′
OXA-48-R	5′ GAGCACTTCTTTTGTGATGGC 3′

using the online Create Assemblies tool with the Unicycler pipeline (Galaxy version 0.5.0 + Galaxy 0). Resistance genes were identified using ABRIcate for mass screening of antimicrobial and virulence genes (Galaxy Version 1.0.1). Replicons were detected using Plasmid Finder (Galaxy Version 2.1.6+galaxy1). The K locus and O serotype were designated with the use of Kaptive (https://kaptive-web.erc.monash.edu/) assessed on 10-11-2024). Integrative conjugative elements were detected with the use of ICEfinder (https://bioinfo-mml.sjtu.edu.cn/ICEfinder/index.php), assessed on 10-11-2024).

RESULTS

The study strain was identified as *K. pneumoniae subspecies pneumoniae.* LFIA and PCR revealed that the strain co-harbored bla_{NDM} and bla_{KPC} genes. The strain carried the bla_{NDM-1} and bla_{KPC-3} genes and was classified as the KL107 O2afg type.

The results of this study focused on analyzing a *K. pneumoniae* strain, designated as GRTHES, isolated from bronchial secretion of an ICU patient in Greece. The study revealed that the strain exhibited extensive antibiotic resistance, particularly to all beta-lactams, including combinations with new agents such as meropenem/vaborbactam and imipenem/relebactam, as well as resistance to aminoglycosides, fluoroquinolones, fosfomycin, trimethoprim-sulfamethoxazole and colistin. Tigecycline remained the only active antibiotic against this strain (Table 2).

The genome analysis of *K. pneumoniae* strain GRTHES revealed a genome length of approximately 5.8 million base pairs with a GC content of 56.9% (Table 3).

Table 2. Antibiotic Susceptibility Testing (AST) Results of *K. pneumoniae* strain GRTHES

Antibiotic	MIC value (mg L^{-1})
Colistin (COL DIL)	4
Imipenem	≥16
Meropenem	≥16
Ampicillin/Sulbactam	≥128
Piperacillin-Tazobactam	≥128
Aztreonam	≥64
Cefepime	≥32
Ceftazidime	≥64
Ceftriaxone	≥64
Ceftazidime/avibactam	≥16
Ceftozolane/tazobactam	≥32
Meropenem/vaborbactam (e test)	48
Meropenem/vaborbactam	≥64
Imipenem/relebactam	≥16
Imipenem/relebactam (e test)	16
Amikacin	≥32
Gentamycin	≥16
Ciprofloxacin	≥4
Levofloxacin	≥8
Fosfomycin	≥128
Tigecycline	1
Trimethoprime/sulphamethoxazole	≥320

Table 3. Statistics of genome of *K. pneumoniae* GRTHES

Statistic	Value
# contigs	117
# contigs (≥ 0 bp)	282
# contigs (≥ 1,000 bp)	100
Largest contig	582,891
Total length	5,769,719
Total length (≥ 0 bp)	5,808,650
Total length (≥ 1,000 bp)	5,757,099
N50	270,719
N90	33,140
auN	263,047
L50	8
L90	30
GC (%)	56.9%
Mismatches	–
#N's per 100 kbp	0
#N's	0

Whole-genome sequencing analysis identified multiple resistance genes, including bla_{NDM-1} and bla_{KPC-3}, which confer carbapenem resistance. Additional resistance genes detected included bla_{TEM-1}, bla_{OXA-1}, bla_{SHV-11}, and $bla_{CTX-M-15}$ for beta-lactams; *oqxA* and *oqxB* for fluoroquinolones; *fos*A for fosfomycin; and several aminoglycoside resistance genes (Table 4).

In addition, point mutations linked to antibiotic resistance were observed. Notable mutations included *parC* S80I and *gyrA* S83I conferring high-level resistance to fluoroquinolones, and *mgrB* G37S, which is associated with colistin resistance. A mutation in ompK36 (ompK36_D135DGD) was also noted, which contributes to carbapenem resistance by reducing membrane permeability, further complicating treatment options for infections caused by this strain (Table 5).

The strain was found to harbor four plasmid types — IncFIA(HI1), IncFIB(K), IncFII(K), and IncX3 — that facilitate the horizontal transfer of resistance genes (Table 6). Additionally, virulence factors like siderophore genes (for iron acquisition) and adhesion proteins were identified, highlighting the pathogenic potential of this strain (Table 7). The study's findings underscore the need for vigilant

Table 4. Antibiotic resistance genes of *K. pneumoniae* GRTHES

Antibiotic class	Genes detected
Beta-lactams, Carbapenems	bla_{TEM-1}, bla_{OXA-1}, bla_{SHV-11}, $bla_{CTX-M-15}$ bla_{NDM-1}, bla_{KPC-3}
Aminoglycosides	*aph(3")-Ib, aph(6)-Id, aac(3)-IIe, aac(6')-Ib, aadA2*
Fluoroquinolones	*oqxA, oqxB*
Sulfonamides	*sul1, sul2*
Chloramphenicols	*catA1, catB3*
Trimethoprim	*dfrA12, dfrA14*
Bleomycin	*ble*
Macrolides	*mph*(A)
Quaternary Ammonium Compounds	*qacEdelta1*
Fosfomycin	*fosA*

Table 5. Point mutations linked to antibiotic resistance in *K. pneumoniae* GRTHES

Mutation	Gene	Resistance type
parC S80I	*parC*	Fluoroquinolone
gyrA S83I	*gyrA*	Fluoroquinolone
mgrB G37S	*mgrB*	Colistin
ompK36 D135DGD	*ompK36*	Carbapenem
pmrB R256G	*pmrB*	Colistin

genomic surveillance and tailored therapeutic approaches to combat MDR pathogens like *K. pneumoniae* GRTHES.

The strain also contained various insertion sequences and transposons, which play a significant role in facilitating gene mobility and horizontal gene transfer. Specifically, 10 insertion sequences were identified, including IS26 (linked with the IS6 family), ISKpn14, ISKpn18, ISKpn24, ISKpn28, ISKpn43, IS6100, ISEcl1, ISEc9, and ISVsa5. Five integrative conjugative elements were detected. These elements contribute to the mobility and expression of resistance genes, enhancing the strain's ability to acquire and maintain resistance traits (Table 8).

DISCUSSION

NDM-1 and KPC-2 producing *K. pneumoniae* strains of sequence types ST11 and ST15 have been reported sporadically in China [8–10]. In 2022, Wei D and her team suggested through plasmid transfer assays and phylogenetic analysis that KPC-2 and NDM-1 producing carbapenem-resistant *K. pneumoniae* likely evolved from a KPC-2 producing *K. pneumoniae* progenitor that later acquired a highly transferable $bla_{\text{NDM-1}}$ gene [9]. In the current study, we hypothesize that an NDM plasmid may have been acquired from an ST512 KPC-producing *K. pneumoniae* strain.

In 2024, Posteraro B and colleagues described five NDM-1 and KPC-3 ST512 strains, with the IncX3 plasmid detected in one isolate [11]. The unusual detection of the $bla_{\text{KPC-3}}$ gene in Greece was first reported by Chatzidimitriou et al. in an *Escherichia coli* ST410 strain from Volos [12]. In the *K. pneumoniae* GRTHES strain of this study, four plasmid types were identified: IncFIA(HI1), IncFIB(K), IncFII(K), and IncX3. These plasmids, especially the IncF group, are known for mobilizing transposons such as Tn4401, which carries the bla_{KPC} gene.

The clinical significance of this study lies in the limited treatment options available for managing this strain. The only remaining active antibiotic was tigecycline which according to the Federal organization of Drugs is indicated for complicated skin and skin structure Infections, complicated Intra-abdominal Infections and community-acquired bacterial pneumonia [13].

Table 7. Virulence factors of *K. pneumoniae* GRTHES

Virulence Factor	Description
ecpA, ecpB, ecpC, ecpD, ecpE	*Escherichia coli* common pilus structural components
fyuA	Yersiniabactin receptor protein
ybtE, ybtT, ybtU, irp1, irp2, ybtA, ybtP, ybtQ, ybtX, ybtS	Yersiniabactin siderophore biosynthesis components
ompA	Outer membrane protein A
fepC, entB, entA	Enterobactin synthesis and transport

Table 8. Transposons and Insertion sequences of *K. pneumoniae* GRTHES

MGE type	MGE name	Family	Synonyms	Accession number
Transposon	Tn5403	Tn	None	X75779.1
Transposon	Tn4401	Tn3	Tn4401c, Tn4401a, Tn4401b	KT378596.1
Insertion Sequence	IS26	IS6	IS160, IS26L, IS26R, IS6, IS140, IS46	X00011
Insertion Sequence	ISKpn14	IS1	None	CP000649
Insertion Sequence	ISKpn18	IS3	None	CP003200
Insertion Sequence	ISKpn24	IS66	None	NC_014312
Insertion Sequence	ISKpn28	IS481	None	NC_009649
Insertion Sequence	ISKpn43	IS110	IS1111	CP024839
Insertion Sequence	IS6100	IS6	IS6100R, IS6100L	X53635
Insertion Sequence	ISEcl1	IS3	IS2	AF342826
Insertion Sequence	ISEc9	IS1380	ISEcp1, ISEcp1B	AJ242809
Insertion Sequence	ISVsa5	IS4	IS10R	NC_011312

Limitations of the study

The study on the antibiotic resistance profile of the *K. pneumoniae* strain GRTHES has several limitations.

Table 6. Plasmid replicons identified in *K. pneumoniae* GRTHES

Plasmid type	Identity (%)	Length of coverage	Node	Plasmid reference ID
IncFIA(HI1)	98.45	387/388	NODE_51_length_**10128**_cov_217.496	HI1
IncFIB(K)	100	560/560	NODE_45_length_**12720**_cov_185.456	Kpn3
IncFII(K)	100	148/148	NODE_35_length_**23717**_cov_170.352	CP000648
IncX3	100	374/374	NODE_61_length_**5994**_cov_367.025	JN24785

Firstly, it examines only a single strain, which may not capture the full diversity of resistance mechanisms in other strains of *K. pneumoniae*. The study relies mainly on genomic sequencing and bioinformatics, which, while robust, depend on existing databases that may not account for novel resistance genes. Additionally, the laboratory conditions under which susceptibility testing was performed might not accurately reflect the complex interactions in clinical environments. Finally, further clinical studies are needed to validate the in vitro findings and assess their implications for patient care. Addressing these limitations is essential for a deeper understanding of the clinical and epidemiological impact of multidrug-resistant *K. pneumoniae*.

CONCLUSION

The findings from this study underscore the urgent need to address the rising tide of antibiotic-resistant *K. pneumoniae* strains, particularly the identified strain GRTHES, which poses significant challenges due to its extensive resistance profile. The genomic insights obtained reveal critical information about the mechanisms underlying multidrug resistance and hypervirulence, signaling a shift in the bacterial landscape. As this strain displays susceptibility only to colistin, the reliance on limited treatment options raises concerns for public health. Therefore, it is imperative to enhance surveillance efforts, promote responsible antibiotic use, and invest in the development of innovative therapeutic strategies to effectively manage these formidable pathogens. Continued research and collaboration among healthcare professionals, microbiologists, and policymakers will be essential in combating the threat of antibiotic resistance and ensuring improved patient outcomes.

Author contributions: CM: conceptualization methodology and design of the study, resources, data curation, writing—original draft preparation, writing—review and editing; TP: writing-original draft preparation; AV: Laboratory testing, drafting the article or revising it critically for important intellectual content, writing; MAK: software, validation, formal analysis; FC: Laboratory testing; MM: Laboratory testing;

SV: writing, laboratory testing; MS: writing; EV: final approval of the version to be submitted. All authors have read and agreed to the published version of the manuscript.

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