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RESEARCH ARTICLE

First detection of a colistin-resistant *Klebsiella aerogenes* isolate from a critically ill patient with septic shock in Bulgaria

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ABSTRACT

Colistin is considered as the last-line antibiotic for the treatment of infections caused by extensively drug-resistant Gram-negative pathogens belonging to the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) group. The present study aimed to explore the colistin resistance mechanisms of a *Klebsiella aerogenes* (formerly *Enterobacter aerogenes*) isolate (Kae1177-1bg) obtained from a Bulgarian critically ill patient with septic shock in 2020. Antimicrobial susceptibility testing and whole-genome sequencing using DNA nanoball technology were performed. The resulting read pairs were used for draft genome assembly, MLST analysis and mutation screening in the *pmrA*/*B*, *phoP*/*Q*, and *mgrB* genes. Kae1177-1bg demonstrated high-level resistance to colistin, resistance to 3rd generation cephalosporins and susceptibility to all other antibiotics tested. In our strain a CMY-2-type class C cephalosporinase was the only β-lactamase identified. No mobile colistin resistance (*mcr*) genes were detected. A total of three missense variants in the genes for the two-component PmrA/PmrB system were identified. Two of them were located in the *pmrB* (pR57K and pN275K) and one in the *pmrA* gene (pL162M). The pN275K variant emerged as the most likely cause for colistin resistance because it affected a highly conservative position and was the only nonconservative amino acid substitution. In conclusion, to the best of our knowledge, this is the first documented clinical case of a high-level colistin-resistant *K. aerogenes* in Bulgaria and the first identification of the nonconservative amino acid substitution pN275K worldwide. Colistin-resistant Gram-negative pathogens of ESKAPE group are serious threat to public health and should be subjected to infection control stewardship practices.

KEYWORDS

colistin resistance, ESKAPE, *Klebsiella aerogenes*, whole-genome sequencing, *pmrB* mutation

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INTRODUCTION

Antimicrobial-resistant ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) pathogens represent a substantial therapeutic problem worldwide [1]. They frequently "escape" from the most commonly used antimicrobial treatment via acquisition and/or development of multiple resistance mechanisms [2]. Therefore, the World Health

Organization designated them critical or high priority status on its list for research and development of new antibiotics for antibiotic-resistant bacteria [3]. Colistin, also known as polymyxin E, is considered as a last-line therapy for infections caused by extensively drug-resistant (XDR) Gram-negative ESKAPE bacteria (non-susceptible to at least one agent in all but two or fewer antimicrobial categories) [4]. However, since 2015, its efficiency has been largely compromised by the emergence and rapid dissemination of mobile colistin resistance (*mcr*) genes among *Enterobacterales* species worldwide [5]. In contrast, no *mcr* determinants were detected among clinical *Klebsiella* species isolates on the Balkan Peninsula. The corresponding molecular mechanisms in these strains have still not been studied in detail.

The present study aimed to explore the colistin resistance mechanisms of the first *Klebsiella aerogenes* (formerly *Enterobacter aerogenes*) isolate found in Bulgaria, which was obtained from a critically ill patient with septic shock.

MATERIALS AND METHODS

Clinical case presentation

The *K. aerogenes* Kae1177-1bg isolate was obtained at the end of May 2020 from the blood sample of a 72-year-old female with acute subarachnoid and intraventricular hemorrhage. The blood culture was also positive for XDR *A. baumannii*, susceptible only to colistin. The patient had been admitted to the Clinic of Neurosurgery at "St. Ivan Rilski" University hospital in Sofia, where she underwent endovascular embolization of two cerebral aneurysms (one on the anterior cerebral artery and the other on the middle cerebral artery), 3 weeks prior to the isolation of Kae1177-1bg. After the surgical procedure she was transferred to the Intensive Care Unit (ICU) with stable vital signs and no newly developed neurological deficit. The early postoperative period was complicated by a worsening hydrocephalus which required the placement of an external ventricular drainage. During the prolonged stay in the ICU, the patient's condition deteriorated and became critical, which was consistently manifested by gallbladder hydrops, development of liver failure, sepsis, and respiratory failure. The patient was intubated and placed on volume-controlled mechanical ventilation. Two days later she died of septic shock and multiple organ failure. Intravenous broad-spectrum antimicrobial therapy including meropenem, vancomycin, and fluconazole, was performed until the patient's death.

Species identification of the isolates

Species identification of the isolates was done using the BBL Crystal Enteric/Nonfermenter ID Kit (Becton Dickinson, Franklin Lakes, NJ). Then the Kae1177-1bg identification was confirmed by analyzing the assembled draft genome sequence using the Microbial Genomes Atlas (MiGA) Web server [6]. The included workflow for the NCBI Genome Database, Prokaryotic section was followed with default settings.

Antimicrobial susceptibility testing

The antimicrobial susceptibility of the studied *K. aerogenes* isolate was determined by the minimum inhibitory concentration (MIC) gradient test (MIC Test Strip; Liofilchem, Roseto degli Abruzzi, Italy) or the broth microdilution method (MICROLATEST MIC Colistin; Erba Lachema s.r.o., Brno, Czech Republic) according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations [7] to the following antimicrobial agents: ceftriaxone, ceftazidime, ceftazidime-avibactam, cefepime, imipenem, meropenem, amikacin, gentamicin, levofloxacin, tigecycline, trimethoprim-sulfamethoxazole, and colistin.

Whole-genome sequencing

The whole-genome sequencing was performed using DNA nanoball sequencing technology as previously described [8]. In brief, genomic DNA isolated from Kae1177-1bg was randomly size fragmented using a Covaris g-TUBE device, and fragments were size selected by magnetic beads to an average size of 200–400 bp. The purified fragments were end repaired, 3′-adenylated, ligated to adapters, and then PCR amplified. The final generated library had an insert size of 350 bp, and it was loaded onto an MGISEQ-2000 platform (BGI Group, Hong Kong, China). There the sequencing step was done generating 2 × 150-bp paired-end reads.

Draft genome assembly

All steps of quality control, raw reads preprocessing, and draft genome assembly were carried out through the Galaxy online platform [9]. Default parameters were used for all following software tools unless otherwise specified. The entire procedure was performed as previously described [8] using the following tools: FastQC v0.11.9 [10], Trimmomatic v0.38 [11], and SPAdes v3.12.0 [12]. The genome completeness level and the closest match by average nucleotide identity (ANI) among all complete and chromosome-level genomes were assessed by the MiGA Web server [6].

Identification of β-lactamase (*bla*) genes, colistin resistance determinants and chromosomal mutations

The draft genome contigs and the raw sequencing reads were screened for *bla* and *mcr* genes using the ResFinder 4.1 tool [13]. Colistin resistance-related mutations in chromosome genes of the Kae1177-1bg isolate were detected by loading the trimmed sequencing read pairs into the Snippy v.3.2 tool using the genome of *K. aerogenes* ATCC 13048 as a reference strain [14]. The multiple sequence alignment of protein sequences was done through the Clustal Omega tool [15] while the frequency of the identified amino acid substitutions was assessed through the blastp tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Multilocus sequence typing (MLST) analysis

The MLST analysis was performed on the assembled draft genome sequence of Kae1177-1bg using the Multilocus sequence typing tool (Galaxy Version 2.19.0, https://usegalaxy.eu/).

RESULTS

Two ESKAPE group isolates were obtained from the blood sample of our critically ill patient with septic shock – an XDR *A. baumannii*, susceptible only to colistin, and the *K. aerogenes* Kae1177-1bg which exhibited a colistin resistance phenotype. The detailed antimicrobial susceptibility testing revealed that Kae1177-1bg demonstrated a rare antimicrobial resistance profile: high-level resistance to colistin, resistance to 3rd generation cephalosporins and susceptibility to all other antibiotics tested (Table 1).

The performed whole-genome sequencing generated 5,175,973 raw read pairs that were next used for the *de novo* draft genome assembly of Kae1177-1bg. It was comprised by 45 contigs larger than 1,000 bp (largest contig – 713,991 bp) with total size of 5.29-Mb, an N50 value of 290,328 and an average GC content of 54.77%. The genome completeness level was assessed to be 97.2%. The closest relative found in the available databases was *K. aerogenes* Ka37751 (GenBank accession no. CP041925.1; 98.75% ANI).

The multilocus sequence typing analysis determined Kae1177bg to be a novel sequence type (ST) with an allelic profile: *dnaA* (24) – *fusA* (2) – *gyrB* (1) – *leuS* (14) – *pryG* (8) – *rplB* (6*) – *rpoB* (8) because of the novel allele that was found in the *rplB* locus.

A CMY-2-type class C cephalosporinase was detected. Its sequence was 75.22% identical to the $bla_{\text{CMY-108}}$ gene (GenBank accession no. KF564648). No extended-spectrum β-lactamases were found.

Table 1. Antimicrobial resistance profile of the *K. aerogenes* Kae1177-1bg isolate

Antimicrobial agent	Minimum Inhibitory Concentration (mg L^{-1})	Interpretation
Colistin	>16	Resistant
Ceftriaxone	32	Resistant
Ceftazidime	64	Resistant
Ceftazidime-avibactam*	0.094	Susceptible
Cefepime	0.064	Susceptible
Imipenem	0.25	Susceptible
Meropenem	0.50	Susceptible
Amikacin	0.75	Susceptible
Gentamicin	0.25	Susceptible
Levofloxacin	0.032	Susceptible
Tigecycline	0.25	Susceptible
Trimethoprim-sulfamethoxazole (1:19)	0.50	Susceptible

*The concentration of avibactam is fixed at 4 mg L^{-1}

None of the known *mcr* genes were detected in Kae1177-1bg when the draft genome contigs and the raw sequencing reads were screened for their presence. Three missence variants were detected when the coding sequence of the *pmrA, pmrB, phoP, phoQ,* and *mgrB* genes of the Kae1177-1bg isolate was screened. They are shown in Fig. 1 (A and B). Two of them were located in the *pmrB* gene (pR57K and pN275K) and one in the *pmrA* gene (pL162M).

Multiple sequence alignment of the PmrA and PmrB protein sequences from five different *Enterobacterales* species (*K. aerogenes*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Escherichia coli* and *Salmonella enterica*) revealed that one of the variants (pR57K in PmrB) affected non-conservative amino acid position while in contrast, the pN275K variant in the coding sequence of the same gene lied in a highly conservative region (Fig. 1-B).

Search in the available protein databases at NCBI, made to assess the frequency of the identified in this study amino acid substitutions indicated that only pR57K was found in deposited isolates and the remaining two variants were not detected so far.

Our mutation screening also identified that the Kae1177-1bg isolate harbored three sequence variants in the *mgrB* gene, located in close proximity upstream of the start codon (Fig. 1-C).

DISCUSSION

To date, only colistin-resistant *K. pneumoniae* clinical isolates have been found in Bulgaria [16–18]. The first four isolates were recovered in August 2016 from samples of two ICU patients hospitalized at the Military Medical Academy in Sofia [16]. They showed identical antimicrobial susceptibility profiles (carbapenem- and multidrug-resistant) and exhibited a high-level resistance to colistin (MIC >16 mg L^{-1}). The isolates possessed KPC-2 carbapenemase and SHV-5 extended-spectrum β-lactamase. No plasmid-mediated *mcr-1* genes were identified. Newer studies reported an incidence of 34%–37% of colistin-resistant strains among carbapenemase-positive *K. pneumoniae* (predominantly NDM metallo-β-lactamase-producing) isolated during the period 2013–2018 from patients of Alexandrovska University Hospital in Sofia [17, 18]. A recent study comparing the *in vitro* activities of the polymyxins against multidrug-resistant Gram-negative ESKAPE isolates from Turkish inpatients (2017–2021) reported 18.5% of colistin-resistant *K. pneumoniae* [19]. Also, no plasmid-borne *mcr* genes were detected in the isolates regardless that these determinants are known as the dominant colistin resistance mechanism among *Enterobacterales* species worldwide [5, 20].

The Kae1177-1bg isolate was a high-level colistin-resistant *K. aerogenes* without *mcr* genes identified. The only other clinical colistin-resistant *K. aerogenes* isolate detected on the Balkan Peninsula was recovered from a 71-year-old male patient in Croatia [21]. Once again, no *mcr*

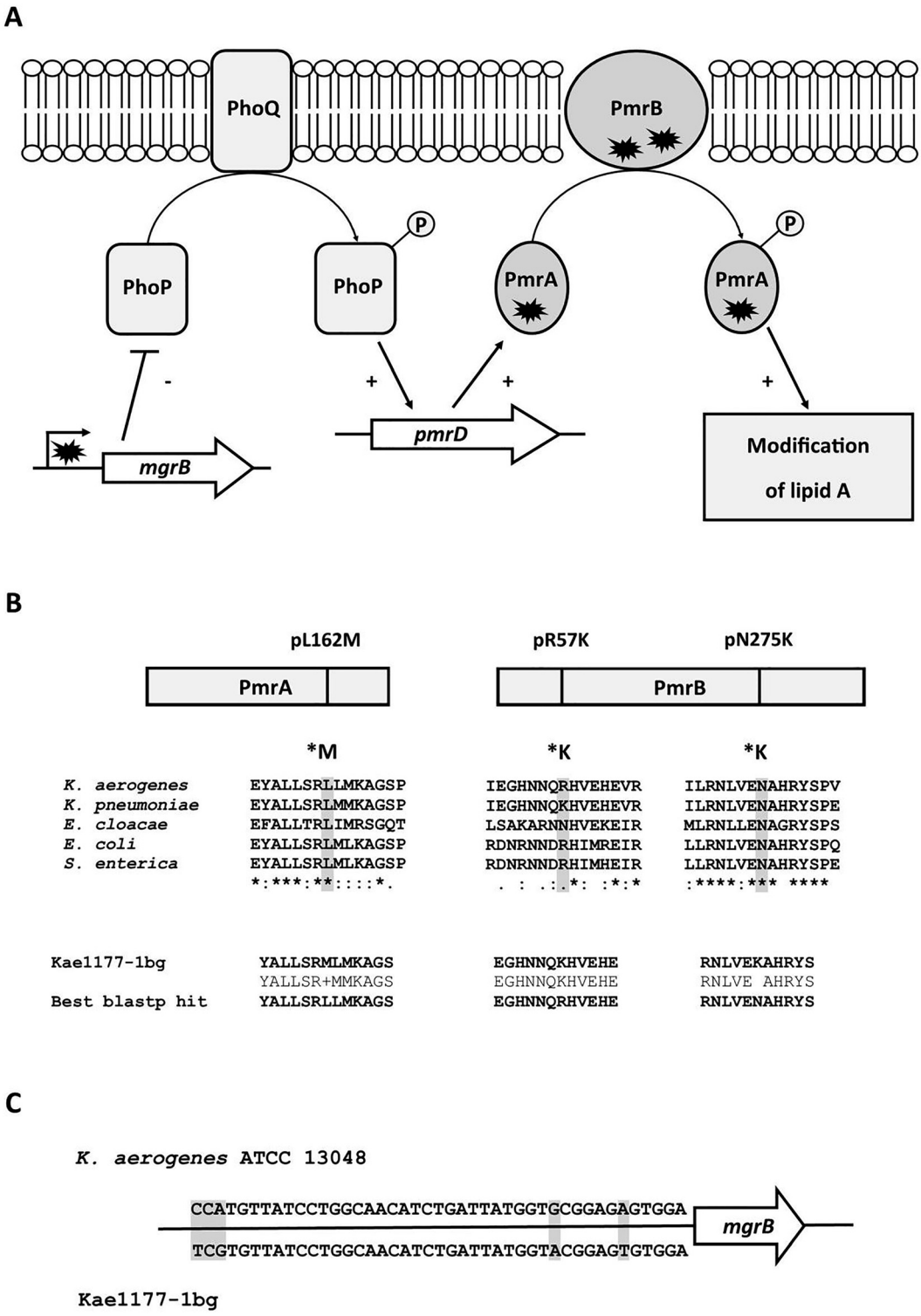

Fig. 1. Identified gene variants involved in the Two-Component Systems (TCS) PhoPQ and PmrAB of the *K. aerogenes* Kae1177-1bg isolate (A) Major compounds of the signaling cascade that activates lipopolysaccharide-modifying genes involved in polymyxin resistance in Gram-negative bacteria. All identified sequence variations in Kae1177-1bg are denoted by black-colored star symbols; (B) A detailed overview of all identified amino acid substitutions including their positions in the coding sequences of the *pmr* genes, multiple sequence alignment of these regions in five different Enterobacteriaceae species (Clustal Omega tool; UniProtKB: *K. aerogenes* – A0A3S4EP52_KLEAE/A0A8A7BQB6_KLEAE, *K. pneumoniae* – A0A483MY47_KLEPN/A0A6G9RV95_KLEPN, *E. cloacae* – A0A0M7DYF8_ENTCL/A0A7D3UL57_ENTCL, *E. coli* – P30843/P30844, and *S. enterica* – A0A3Y0F0L9_SALET/A0A0D3MMQ9_SALET), and the best blast hits against the database Microbial_proteins (blastp tool, default settings). All given amino acid positions are numbered according to the corresponding sequences in *K. aerogenes* ATCC 13048; (C) Identified variants upstream of the coding sequence of the *mgrB* gene

determinants were found suggesting that the most common colistin resistance mechanisms observed on the Balkans are chromosomal-mediated. They could include mutations in different chromosome genes, changes in the bacterial cell outer membrane and/or the presence of an efflux pump. Unfortunately, the PCR-based screenings used by the authors were unable to provide further insights into their molecular foundations.

The applied whole-genome sequencing approach here allowed us to screen Kae1177-1bg for mutations in the *pmrA*/*pmrB*, *phoP*/*phoQ* and *mgrB* genes that were affected in the majority of the studied cases of chromosomal-mediated colistin resistance in *Enterobacterales* members [22]. Three missense genetic variants found in the *pmrA* and *pmrB* genes were the most likely cause for the colistin resistance in Kae1177-1bg. This finding was further supported by the observed antimicrobial resistance profile of the isolate. It excluded the various adaptive mechanisms as a possible explanation since they are expected to influence the susceptibility not only to polymyxins but also to other antibiotics.

The strongest candidate among these missense mutations was the pN275K variant in the coding sequence of the *pmrB* gene because it affected a highly conservative position and was the only nonconservative amino acid substitution identified.

Having in mind that approximately one third of the recently reported clinical colistin-resistant *K. pneumoniae* isolates from Bulgaria showed changes in the *mgrB* gene [18], we analyzed the identified non-coding variants in its promotor region for possible role in the colistin resistance phenotype. Variants upstream of the translation start site can suppress the gene's expression and various loss of function mutations in *mgrB* have been recovered from many colistin-resistant clinical strains until now [23]. Despite that, it is not likely that these non-coding variants caused the resistance phenotype in our isolate because we detected the same haplotype in several other *K. aerogenes* isolates and none of them was reported to be colistin-resistant. This finding suggested that *mgrB* mutations are not a leading cause for colistin-resistance in *K. aerogenes* in contrast to their role in *K. pneumoniae*. Moreover, it was supported by the colistin-resistant *K. aerogenes* isolate from Croatia, which was also negative for mutations in this gene [21].

The only other clinically important finding from the antimicrobial susceptibility testing of Kae1177-1bg was the observed resistance to 3rd generation cephalosporins. It can be explained by the detection of a CMY-2-type AmpC β-lactamase, which was most similar to the CMY-108. As it is well known, certain mutations are responsible for stable derepression of the *ampC* gene and these are frequent cause of clinical resistance to all beta-lactams apart from carbapenems and sometimes cefepime [24]. High mutation rates (a mean value of 3×10^{-8}) have been recently reported in some *Enterobacterales* species with chromosomally encoded inducible AmpC β-lactamase including *K. aerogenes* [25].

In conclusion, the current study demonstrates that colistin-resistant *K. aerogenes* isolates have already emerged in Bulgaria. To the best of our knowledge, this is the first documented clinical case of a high-level colistin-resistant *K. aerogenes* in the country. Whole-genome sequencing reveals that our isolate lacks *mcr* genes and utilizes a chromosomal-mediated resistance mechanism presumably due to the pN275K mutation found in the *pmrB* gene. Also, this is the first report of the nonconservative amino acid substitution pN275K worldwide. The observed antimicrobial resistance profile does not highlight Kae1177-1bg as a formidable challenge for treatment given its susceptibility to all antibiotics tested except for colistin and 3rd generation cephalosporins. Nevertheless, our detected co-infection with an XDR *A. baumannii*, susceptible only to colistin, shows that the colistin resistance in analogous cases can be a serious threat to public health, and therefore such isolates should be subject to infection control stewardship practices in hospitals.

Nucleotide sequencing

Whole-genome shotgun sequencing project of the *K. aerogenes* Kae1177-1bg isolate has been deposited in GenBank under Accession no. JAJKKF000000000.1.

Conflict of interest: The authors report no conflicts of interest.

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