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RESEARCH

Molecular characterization of colistin resistance genes in a high-risk ST101/KPC-2 clone of *Klebsiella pneumoniae* in a University Hospital of Split, Croatia

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Abstract

Klebsiella pneumoniae carbapenemase-producing *K. pneumoniae* (KPC-KP) has become a major concern worldwide due to multidrug resistance and the ability to spread locally and globally. Infections caused by KPC-KP are great challenge in the healthcare systems because these are associated with longer hospitalization and high mortality. The emergence of colistin resistance has significantly reduced already limited treatment options. This study describes the molecular background of colistin-resistant KPC-KP isolates in the largest hospital in southern Croatia. Thirty-four non-duplicate colistin-resistant KPC-KP isolates were collected during routine work from April 2019 to January 2020 and from February to May 2021. Antimicrobial susceptibility was determined using disk diffusion, broth microdilution, and the gradient strip method. Carbapenemase was detected with an immunochromatographic test. Identification of bla_{KPC} and *mcr* genes or mutations in *pmrA*, *pmrB*, *mgrB*, *phoP*, and *phoQ* genes were performed by polymerase chain reaction (PCR) and positive products were sequenced. Pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) were used for epidemiological analysis. All isolates were multidrug-resistant, with colistin minimum inhibitory concentrations (MICs) from 4 to >16 mg/L, and all harbored bla_{KPC-2} and had a single point mutation in the *mgrB* gene resulting in a premature stop codon, with the exception of one isolate with four point mutations corresponding to stop codons. All isolates were negative for *mcr* genes. PFGE analysis identified a single genetic cluster, and MLST revealed that all isolates belonged to sequence type 101 (ST101). These results show emergence of the high-risk ST101/KPC-2 clone of *K. pneumoniae* in Croatia as well as appearance of colistin resistance due to mutations in the *mgrB* gene. Molecular analysis of epidemiology and possible resistance mechanisms are important to develop further strategies to combat such threats.

Keywords *Klebsiella pneumoniae* · KPC · ST101 · Colistin resistance · *mgrB* mutation

Introduction

The management of life-threatening infections caused by multidrug-resistant *Klebsiella pneumoniae* has become a considerable challenge worldwide (Bassetti et al. 2018). This species easily acquires resistance to different groups of antibiotics through the transfer of genetic elements and/or accumulation of chromosomal mutations (Holt et al. 2015). Extensive use of carbapenems has led to the emergence of carbapenem-resistant strains, among which *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae* (KPC-KP) is one of the most potent producers regarding the range of the hydrolysis spectrum and ability to spread (Nordmann et al. 2012). The KPC enzyme belongs to Ambler class A carbapenemases and hydrolyzes a broad variety of β-lactams,

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including penicillins, cephalosporins, carbapenems, and aztreonam (Nordmann et al. 2012). According to a systematic review of the scientific literature and meta-analysis of all studies that reported KPC-KP infection-related mortality, performed by Ramos-Castañeda et al. (2018), KPC-KP infections are highly lethal, with a mortality rate of 40%, and have increased in recent years. The epidemiological distribution of KPC-KP sequence types (ST) is continuously changing in favor of those with greater epidemiological potential (Del Franco et al. 2015, Roe et al. 2019; Loconsole et al. 2020), some of which have shown a high number of virulence genes (Oteo et al. 2016; Roe et al. 2019).

Emergence of resistance to colistin, which is often one of the very few therapeutic choices in the treatment of such infections, entails the need for early detection of colistin resistance, especially in areas of increased use. In addition, the study of colistin resistance mechanisms is important for developing infection control and antimicrobial stewardship strategies.

Colistin (polymyxin E) is a polypeptide antibiotic from a class of polymyxins that destabilizes lipopolysaccharide (LPS) on the outer membrane of gram-negative bacteria, causing increased permeability of the membrane and leakage of the cytoplasmic content, which leads to death of bacterial cells (Poirel et al. 2017). Additionally, polymyxins bind to LPS during cell lysis and thus neutralize it and inhibit bacterial respiratory enzymes in the inner membrane (Poirel et al. 2017).

In *K. pneumoniae*, acquired polymyxin resistance is mostly mediated by mutations/changes in chromosomal genes, although plasmid-mediated resistance has a tendency for horizontal dissemination and has been reported worldwide (Poirel et al. 2017; Berglund 2019). Chromosomal changes can affect many different genes and operons responsible for the synthesis and/or addition of cationic groups (4-amino-4-deoxy-l-arabinose (L-Ara4N) or phosphoethanolamine (pEtN)) to LPS, thereby modifying it in a way that decreases the affinity of polymyxins to the LPS target (Poirel et al. 2017). Inactivation of the *mgrB* gene seems to be the most common mechanism of colistin resistance among KPC-KP (Cannatelli et al. 2013, 2014; Olaitan et al. 2014; Cheng et al. 2015; Giani et al. 2015; Poirel et al. 2015; Zowawi et al. 2015; Bathoorn et al. 2016; Haeili et al. 2017; Leung et al. 2017; Giordano et al. 2018; Di Tella et al. 2019; Xu et al. 2020; Rocha et al. 2022). Numerous other mechanisms of resistance to colistin have also been described, many of which have yet to be fully elucidated (Poirel et al. 2017; Berglund 2019).

The product of the *mgrB* gene is the small transmembrane MgrB protein of 47 amino acids that is a negative regulator of the PhoPQ two-component signaling system (PhoQ protein with tyrosine kinase activity and PhoP protein that is activated by PhoQ through phosphorylation). When the regulation is inhibited due to inactivated MgrB protein, the unrepressed *phoPQ* operon causes PhoP to activate the transcription of the *pmrHFIJKLM* operon, leading to the addition of L-Ara4N to LPS (Poirel et al. 2017). In addition, PhoP can activate the PmrA protein, leading to the addition of pEtN to LPS (Poirel et al. 2017).

The aims of this study were to investigate the epidemiological background of colistin-resistant KPC-KP isolates collected from inpatients at the University Hospital of Split (UHS) during two separate time periods, to observe potential epidemiological fluctuations, to characterize the variant of the KPC enzyme, and to investigate possible molecular mechanisms of colistin resistance.

Materials and methods

Study isolates

A total of 34 non-duplicate colistin-resistant KPC-KP isolates were collected from clinical and screening samples in the UHS during 2 collection periods: from April 2019 to January 2020 and from February to May 2021. All isolates are part of the outbreaks in the UHS, with the index case in August 2018. The first collection period was before and the second after the implementation of strong coronavirus disease 2019 (COVID-19) outbreak control measures in March 2020 (lockdown and reduction of hospital activities), during which we had a short time without newly discovered cases. One of the intentions of the second collection period was to observe any changes in the epidemiology and resistance mechanisms of KPC-KP isolates in the UHS.

Identification of the species level was performed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF/MS) (Bruker Daltonics GmbH, Bremen, Germany). The type of carbapenemase was phenotypically detected with an immunochromatographic test (ICT RESIST-4 O.K.N.V. K-SeT, Coris BioConcept, Gembloux, Belgium).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of isolates was performed by the disk diffusion method for ampicillin (10 µg), amoxicillin/clavulanic acid (20/10 µg), piperacillin/tazobactam (30/6 µg), cefuroxime (30 µg), cefotaxime (5 µg), ceftriaxone (30 µg), ceftazidime (10 µg), cefoxitin (30 µg), cefepime (30 µg), ceftazidime/avibactam (10/4 µg), ceftolozane/tazobactam (30/10 µg), imipenem (10 µg), meropenem (10 µg), ertapenem (10 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), gentamicin (10 µg), and amikacin (30 µg) (Mast Group, UK). Minimum inhibitory concentrations (MICs) were detected

by the gradient strip method for fosfomycin (BioMerieux) and the broth microdilution method for colistin (Microlatest MIC Colistin, Erba Lachema, Czech Republic). Interpretative criteria were based on European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoint tables, version 11.0.

Molecular epidemiology

The genetic relatedness of the studied isolates was assessed by pulsed-field gel electrophoresis (PFGE) of *Xba*I-digested genomic DNA using the CHEF-DR III System (Bio-Rad Laboratories, USA) as described previously (Jelic et al. 2015). PFGE band pattern similarity calculation and clustering were performed using the Dice coefficient (1.8% tolerance) and unweighted pair group method with arithmetic mean (BioNumerics v7.6, Belgium). Isolates with ≥85% similarity were assigned to the same cluster. Multilocus sequence typing (MLST) was performed according to the protocol described on the *K. pneumoniae* MLST website (https://bigsdb.pasteur.fr/klebsiella/primers-used/).

Molecular characterization of carbapenemase genes

The presence of KPC-encoding genes was confirmed by polymerase chain reaction (PCR) (Jelic et al. 2015). Sequencing of PCR products was performed on a 3500 Genetic Analyzer using BigDye 3.1 technology (Thermo Fisher Scientific, USA).

Molecular characterization of colistin resistance genes

Detection of genes conferring resistance to colistin was also performed by PCR for (Bassetti et al., 2018) plasmid genes *mcr-1*, *mcr-2*, *mcr-3*, and *mcr-4* and (Bathoorn et al., 2016) chromosomal genes *mgrB, pmrA*, *pmrB*, *phoP*, and *phoQ* (Haeili et al. 2017). Sequencing of chromosomal genes was performed to assess their primary DNA structure. Mutations in the genes were identified by comparison with the nucleotide sequences of the wild-type genes taken from the GenBank database (www.ncbi.nlm.nih.gov/genbank/) using the Clustal Omega web tool for multiple sequence alignment (https://www.ebi.ac.uk/Tools/msa/clustalo/). Accession numbers of the DNA sequences of the reference WT *phoP*, *phoQ*, *pmrA*, *pmrB*, and *mgrB* genes were as follows: MG243705 to MG243721, MF431844, and MF431845. Translation of the *mgrB* gene DNA sequence was performed by the Expasy Translate Tool (https://web.expasy.org/translate/).

Results

Study isolates

Among 34 collected colistin-resistant KPC-KP isolates, 26 were collected in the first collection period, and 8 were collected in the second collection period (Table 1).

Thirty isolates were from clinical samples, and 4 were from screening samples (rectal swabs) (Table 1). The clinical samples were isolated from urine (n=16), blood (n=10), lower respiratory tract (n=2), deep wound (n=1), and central venous catheter (n=1) (Table 1). The samples were mostly obtained from patients in intensive care units (n=15), nephrology (n=7), pulmonology (n=4), and urology (n=4) (Table 1).

The KPC-type carbapenemase was detected with a rapid immunochromatographic test in all isolates.

Antimicrobial susceptibility testing

All isolates had a multidrug-resistant phenotype with high-level resistance to penicillins, cephalosporins, carbapenems, piperacillin/tazobactam, ceftolozane/tazobactam, and fluoroquinolones. The resistance to colistin was expressed with MICs ranging from 4 to >16 mg/L (Table 1). Regarding the other tested antibiotics, all tested isolates except one were susceptible to ceftazidime/avibactam, most remained susceptible to trimethoprim-sulfamethoxazole (74%, n=25), and some were susceptible to tigecycline (26%, n=9), fosfomycin (15%, n=5), gentamicin (12%, n=4), and amikacin (3%, n=1) (Table 1). Isolates from six patients were susceptible to only one antibiotic tested (five to ceftazidime/avibactam and one to tigecycline only).

Molecular epidemiology and characterization of carbapenemase genes

PFGE analysis revealed that all isolates were clonally related, belonged to the same genetic cluster (≥85% similarity), and, according to MLST, were assigned to the ST101 sequence type, suggesting the clonal expansion and cross-transmission of KPC-KP throughout multiple wards within a hospital (Table 1). The presence of bla_{KPC} was detected by PCR in every isolate, and sequencing revealed that they all harbor bla_{KPC-2}.

Molecular characterization of colistin resistance genes

All isolates were negative for the *mcr-1*, *mcr-2*, *mcr-3*, and *mcr-4* genes.

Table 1 Epidemiological data, in vitro susceptibility, and genotypic characteristics of colistin-resistant KPC-producing *Klebsiella pneumoniae* isolates from the University Hospital of Split

Isolate	Date	Sample	Ward	Colistin MIC (mg/L)	Susceptible to:	Clone	Sequence type	KPC type	Type of mgrB mutation	MgrB protein
CR1	Apr 2019	RA	PUL	4	SXT, C/A, TIG*	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR2	Jun 2019	U	URO	16	SXT, C/A	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR3	Jun 2019	B	URO	8	SXT, C/A, FOS	I	ST101	KPC-2	Four point mutations (L9X, I10X, I13X, V26X)	Truncated protein (8 aa)
CR4	Jun 2019	B	NEPH	8	C/A	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR5	Jun 2019	U	INF	8	SXT, C/A	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR6	Jul 2019	B	PUL	>16	SXT, C/A, FOS	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR7	Jul 2019	B	ICU	4	SXT, C/A, FOS	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR8	Jul 2019	RA	PUL	4	SXT, C/A	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR9	Aug 2019	U	ORT	8	SXT, C/A, TIG	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR10	Aug 2019	B	ICU	16	C/A	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR11	Sep 2019	U	ICU	>16	SXT, C/A	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR12	Sep 2019	CVCT	ICU	8	SXT, C/A	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR13	Sep 2019	U	NEPH	8	SXT, C/A, TIG	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR14	Sep 2019	B	ICU	8	SXT, C/A, FOS, TIG	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR15	Sep 2019	U	NEU-SUR	>16	SXT, C/A, GEN	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR16	Sep 2019	RA	NEPH	4	SXT, C/A, TIG	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR17	Oct 2019	B	URO	8	SXT, C/A	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR18	Oct 2019	U	NEPH	4	SXT, C/A	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR19	Oct 2019	U	HEM	16	TGC	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR20	Nov 2019	RA	ICU	>16	SXT, C/A	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR21	Nov 2019	U	NEPH	8	SXT, C/A	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR22	Nov 2019	U	ICU	8	SXT, C/A, GEN	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR23	Nov 2019	U	PUL	16	SXT, C/A	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR24	Dec 2019	U	ICU	>16	C/A, GEN, AMI, TIG	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR25	Dec 2019	U	NEPH	4	SXT, C/A	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR26	Dec 2019	U	ICU	8	SXT, C/A, GEN	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR27	Jan 2020	U	NEPH	>16	SXT, C/A	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR28	Jan 2020	U	URO	>16	SXT, C/A	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR29	Jan 2020	B	ICU	8	SXT, C/A, FOS	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR30	Feb 2021	B	ICU	8	C/A	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR31	Mar 2021	BA	ICU	8	C/A	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR32	Mar 2021	BA	ICU	8	C/A	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR33	Apr 2021	B	ICU	16	C/A, TGC	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)
CR34	May 2021	WS	ICU	16	C/A, FOS, TGC	I	ST101	KPC-2	Point mutation (Q30X)	Truncated protein (29 aa)

*Susceptibility to tigecycline was interpreted according to the EUCAST MIC breakpoints validated for *Escherichia coli* and *Citrobacter koseri*

RS, rectal swab; *U*, urine; *B*, blood; *CVCT*, central venous catheter tip; *BA*, bronchial aspirate; *WS*, wound swab; *PUL*, pulmonology; *URO*, urology; *NEP*, nephrology; *INF*, infectious diseases; *ICU*, intensive care unit; *ORT*, orthopedic surgery; *NEU*, neurosurgery; *HEM*, hematology; *MIC*, minimum inhibitory concentration; *KPC*, *Klebsiella pneumoniae* karbapenemase; *SXT*, cotrimoxazole; *C/A*, ceftazidime/avibactam; *TIG*, tigecycline; *FOS*, fosfomycin; *GEN*, gentamicin; *AMI*, amikacin; *aa*, amino acids; *Q*, glutamine; *X*, stop codon

Sequencing results of the *mgrB* gene identified a single point mutation resulting in a premature stop codon at position 30 leading to the synthesis of a truncated MgrB protein (Table 2). The exception is one isolate with four point mutations at positions 9, 10, 13, and 26, resulting in consequent stop codons as well (Tables 1 and 2).

For the other analyzed genes in representative isolates, we identified mutations with consequent amino acid substitutions in PmrA, pmrB, phoP, and phoQ proteins but with no registered effects on protein function, so they are less likely to be associated with colistin resistance.

Discussion

This study highlights the emergence and clonal dissemination of a high-risk ST101/KPC-2 clone of *K. pneumoniae* in the largest hospital in southern Croatia and the occurrence of colistin resistance within the clone most likely due to point mutations in the *mgrB* gene.

PFGE showed that all analyzed isolates belonged to a single genetic cluster regardless of the collection period, indicating the ongoing clonal endemic spread despite the outbreak control measures. Reoccurrence of the same outbreak clone after the period of no recorded cases shows the epidemiological potential of such a clone. Although previously described KPC-KP isolates in Croatia belonged to ST258 (Jelic et al. 2015), it seems that ST101 has been established as predominant in southern Croatia. The emergence of carbapenem-resistant ST101 isolates has been described in various parts of Italy (Del Franco et al. 2015; Di Tella et al. 2019; Roe et al. 2019; Loconsole et al. 2020). Whole genome sequencing (WGS) of high-risk clones from Spain performed by Oteo et al. (2016) showed that the ST101/KPC-2 clone had the highest number of resistance and virulence genes. As the ST101 clone is associated with rapid spread, high virulence, and increased mortality, additional colistin resistance along with previously present extended spectrum resistance raises further concern (Oteo et al. 2016; Roe et al. 2019, Loconsole et al. 2020). A study performed by Can et al. (2018) found that the ST101 clone, along with ICU stay, was a significant independent predictor of mortality among patients infected with colistin-resistant *K. pneumoniae*.

This study showed that colistin resistance in KPC-KP isolates was not exhibited through the production of plasmid-encoded *mcr-1*, *mcr-2*, *mcr-3*, or *mcr-4* genes. This finding is consistent with previous findings that have rarely reported plasmid-mediated colistin resistance in *K. pneumoniae*, especially in clinical isolates (Berglund 2019). Instead, colistin resistance caused by inactivation of the chromosomal *mgrB* gene due to different alterations is the most commonly described mechanism among KPC-KP isolates. The sequencing of the *mgrB* gene in this study revealed a single point nonsense mutation with an ensuing premature stop codon at position 30 and truncated dysfunctional MgrB protein (Table 2). One isolate was different, although it belonged to the same cluster and had four single point mutations at positions 9, 10, 13, and 26, all corresponding to stop codons (Tables 1 and 2). Insertion sequence (IS) elements were not found.

Because complementation studies with a wild-type *mgrB* allele have demonstrated that susceptibility to colistin can be successfully restored in isolates with mutations or insertional inactivation of *mgrB* (Cannatelli et al. 2014; Esposito et al. 2018; Sisti et al. 2022), we can assume that *mgrB* mutation and consequent MgrB inactivation in isolates from our study are the most likely cause of colistin resistance. Cannatelli et al. (2015) showed that *mgrB* inactivation can occur easily in vitro in *K. pneumoniae* without significant biological cost and is maintained in the absence of selective antimicrobial pressure, emphasizing the importance of such a mechanism in the evolution and persistence of colistin resistance in clinical settings. The colistin resistance rates among KPC-KP isolates from UHS in 2019 and 2020 were 21.8% and 19.3%, respectively. The connection between selective antimicrobial pressure and the development of colistin resistance in clinical settings has been observed in many studies (Cannatelli et al. 2013; Giani et al. 2015; Bathoorn et al. 2016; Leung et al. 2017; Kanwar et al. 2018; Xu et al. 2020). Notably, colistin-resistant KPC-KP isolates began to appear in the UHS during the outbreak of multidrug-resistant *Acinetobacter baumannii* infections that were treated with colistin.

Regarding other genes analyzed in this study that can have a role in the development of colistin resistance mechanisms, we identified point mutations in *pmrA*, *pmrB*, *phoP*, and *phoQ* genes of representative isolates, but consequent amino acid substitutions have no registered effects on protein function; thus, they represent a protein polymorphism and are unlikely associated with colistin resistance.

All isolates showed a multidrug-resistant phenotype, and six isolates from this study were susceptible to only one antibiotic tested (Table 1). This finding is concerning as studies have shown that combination therapy compared to monotherapy is superior for treating multidrug-resistant *K. pneumoniae* infections (Bassetti et al. 2018). In addition, panresistant KPC-KP isolates have been recorded (Bathoorn et al. 2016; Xu et al. 2020).

The colistin MIC range of the studied isolates varied from 4 to >16 mg/L. There are other described mechanisms of polymyxin resistance in addition to those that are involved in the regulatory network controlling chemical modifications on lipopolysaccharide, such as capsular polysaccharide hyperproduction and porin and/or efflux pump activation, which likely contributes to changes in MIC levels (Poirel et al. 2017).

Table 2 DNA sequences of the *mgrB* gene and amino acid sequences of the resulting MgrB proteins for wild type, all isolates with the exception of CR3, and the CR3 isolate

Wild type	DNA	GTGAAAAAATTACGGTGGGTTTTACTGATAGTCATCATAGCAGGCTGCCTGTTGCTGTGGACTCAGATGCTT AACGTAATGTGCGACCAGGATGTTCAGTTTTTCAGCGGCATTTGCACTATTAATAAATTTATTCCGTGG TAA
	Protein	V K K L R W V L L I V I I A G C L L L W T Q Met L N V Met C D Q D V Q F F S G I C T I N K F I P W Stop
All isolates except CR3	DNA	GTGAAAAAATTACGGTGGGTTTTACTGATAGTCATCATAGCAGGCTGCCTGTTGCTGTGGACTCAGATGCTT AACGTAATGTGCGACTAGGATGTTCAGTTTTTCAGCGGCATTTGCACTATTAATAAATTTATTCCGTGG TAA
	Protein	V K K L R W V L L I V I I A G C L L L W T Q Met L N V Met C D Stop D V Q F F S G I C T I N K F I P W Stop
CR3	DNA	GTGAAAAAATACGGTGGGTTTTACTGATAGTCATCATAGCAGGCTGCCTGTTGCTGTGGACTCAGATGCTTA ACGTAATGTGCGACTAGGATGTTCAGTTTTTCAGCGGCATTTGCACTATTAATAAATTTATTCCGTGGTAA
	Protein	V K K Y G G F Y Stop Stop S S Stop Q A A C C C G L R C L T Stop C A T R Met F S F S A A F A L L I N L F R G

Stop, stop codon

In conclusion, the spread of ST101/KPC-2 clone resistant even to last-resort antibiotics is of great clinical concern. This clone showed high virulency and is connected with high mortality. The absence of enough effective antimicrobials causes serious difficulties in treatment. Aggressive infection control measures and effective antimicrobial stewardship are necessary strategies to combat such threats, as well as continuous monitoring of high-risk clones and detection of resistance mechanisms. Further investigations are needed to fully understand the epidemiology of such strains and other possible molecular mechanisms of colistin resistance.

Author contribution The study conception and design were made by Zana Rubic and Marko Jelic. Material preparation, data collection, and analysis were performed by Zana Rubic, Marko Jelic, Silvija Soprek, Maja Tarabene, and Josip Ujevic. Marina Radic and Anita Novak participated in data collection and analysis. Supervision was performed by Ivana Goic-Barisic, Arijana Tambic Andrasevic, and Marija Tonkic. The first draft of the manuscript was written by Zana Rubic and Marko Jelic, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval This was an observational study, and ethics approval is not required according to the Ethics Committee of the University Hospital of Split.

Competing interests The authors declare no competing interests.

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