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Original Article

A novel KPC-166 in ceftazidime/avibactam resistant ST307 *Klebsiella pneumoniae* causing an outbreak in intensive care COVID Unit, Italy

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KPC-166;
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Outbreak;
pKpQIL

Abstract *Introduction:* Aim of the study was the molecular characterization of 21 ceftazidime/avibactam resistant (CZA-R) *Klebsiella pneumoniae* strains, collected in the period October 2021—March 2022 from an Intensive Care COVID Unit in a Northern Italian Hospital. *Methods:* After growth on selective/chromogenic culture media and susceptibility tests assessment, resistance genes content was ascertained for all the isolates by the HybriSpot 12 multiplexing, PCR and Whole-Genome Sequencing (WGS). Clonality was assessed by PFGE and MLST according to the Pasteur scheme. A SNPs-based phylogenetic tree was obtained comparing representative isolates and global genomes. The *bla*KPC gene horizontal transmission was evaluated by conjugation experiments. *bla*KPC-166 was cloned in a pCR2.1 vector and transformed in chemically competent TOP10 cells.

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Results: Sixteen inpatients resulted positive for colonization and/or infection by KPC-producing *K. pneumoniae* (KPC-Kp) strains. The 21 CZA-R KPC-Kp isolates obtained showed MDR phenotype; susceptibility to meropenem was always retained. All the CZA-R KPC-Kp presented a novel *bla*KPC variant, named *bla*KPC-166, showing a single nucleotide substitution (T811C) compared to the *bla*KPC-94; but related to *bla*KPC-2.
Two different pulsotypes were detected: A in 18/21 and B in 1/21 cases, two strains from the same patient being untypable by PFGE. Interestingly, the outbreak was sustained by the high-risk clone ST307, although the ST22, ST6342, ST6418 and ST6811 have also been identified and associated to KPC-166. Worryingly, *bla*KPC-166 could be transferred horizontally and, after cloning, it conferred resistance to CZA.
Discussion: This novel variant confers CZA—resistance and carbapenems susceptibility restoration. As KPC-166 was found expressed by multiple Kp clones, greater efforts should be made to prevent the further dissemination of such strains in Italian clinical settings.

Introduction

Carbapenems are critically important antibiotics in human medicine and are among the major options in the treatment of severe infections caused by multidrug-resistant (MDR) *Enterobacterales*, such as *Klebsiella pneumoniae*.[1,2] However, over the past 15 years their effectiveness has been decreased due to carbapenemase production, efflux pumps and target modifications; in particular, the number of carbapenemase-producing *Enterobacterales* (CPE) has drastically raised.[2,3] Treatment of carbapenem-resistant *Enterobacterales* (CRE) is a challenge in hospitalized patients, especially those who are immunocompromised or critically ill. In recent years, new β-lactams/β-lactamases inhibitors (BLBLIs) combinations, as ceftazidime/avibactam (CZA), have been introduced to face severe infection caused by CRE.

CZA, the first BLBLI available, is approved by Food and Drug Administration (FDA) for the treatment of i) hospital-acquired pneumonia, ii) complicated intra-abdominal infections, iii) complicated urinary tract infections, (iv) severe infections by Gram-negative organisms in patients with limited treatment options.[4] Currently, CZA represents one of the first-line options in the treatment of severe CPE infections, as those caused by KPC-producing *K. pneumoniae*, which is of particular concern due to its global endemicity and high morbidity.[4] However, episodes of CZA resistance have been already reported.[5] The first case of CZA resistance was described in 2015 from a patient with no previous exposure to CZA.[6] Nowadays, CZA resistance is largely spreading in clinical settings with an increased number of reports available in literature [4]. *Enterobacterales* may develop CZA resistance by three main mechanisms: a) amino acid substitutions at critical sites of β-lactamases, b) changes in cell permeability and expression of efflux pumps, and c) target protein mutations (PBP2, PBP3).[5,6] Mutations in class A β-lactamases - as KPC - developing CZA resistance, have been extensively reported.[5,6]

The Ω loop of approximately 15 aa (position 164—179) is an important active site for β-lactamases, also involved in the maintenance of the cyclic structure.[5] Mutations in the Ω loop alter the flexibility of the entire loop, both impair the covalent trapping of the β-lactamases to CZA and can affect the effectiveness of CZA, decreasing the binding of avibactam.[5] In recent years, substitutions occurring in the Ω loop of KPC carbapenemases, especially KPC-2 and KPC-3, have been reported associated to CZA resistance. Among these, the D179Y is the most frequently reported substitution leading to CZA resistance, associated to a ≥4-fold restoration in susceptibility to meropenem.[7,8] Yet, phenotypic resistance to CZA has been shown to be highly heterogeneous, as also mediated by altered porin expression, upregulation of efflux systems, production of mutated AmpC, and increased expression of β-lactamases.[5] Microbiological and molecular characterization of resistant isolates is thus of critical importance in building knowledge on the circulation and possible spread of resistant isolates, especially in critically ill and/or immunocompromised patients. Here, we report an outbreak of CZA-resistant (CZA-R) KPC-producing *K. pneumoniae* (KPC-Kp) which took place in a COVID-19 intensive care unit (COVID-ICU). The aim of the study was to characterize deeply, on a genotypic and phenotypic perspective, the features of the CZA-R KPC-Kp isolates involved in the outbreak.

Methods

Bacterial isolates and antimicrobial susceptibility testing

Clinical isolates of CZA-resistant KPC-producing *K. pneumoniae* obtained from patients admitted to COVID-ICU of the ASST Grande Ospedale Metropolitano Niguarda (Milan, Italy) from October 2021 to March 2022 were included in the study. Bacterial growth was obtained on selective/chromogenic culture media, according to the nature of the original sample (i.e. blood, urine, swabs), processed with the WASPLab® automation. CHROMID ESβL and CHROMID CARBA selective chromogenic media (bioMerieux, Firenze, Italy) were used for the selection of ESβLs/carbapenemase-producing *Enterobacterales* (CPE) on the same lab

automation. Phenotypic and genotypic tests for identification of carbapenemases (KPC, VIM, IMP, NDM, OXA- 48) was performed using the immunochromatographic (IC) assay NG-Test CARBA (NG-Biotech, Guiory, France) and the Xpert Carba-R assay (Cepheid, Milan, Italy). All suspected isolates were identified by MALDI-TOF MS Biotyper system (Bruker). Antimicrobial susceptibility testing (AST) was carried out according to standard internal laboratory protocol using a NMDRM-1 microdilution panel on a MicroScan WalkAway semi-automated system (Beckman Coulter, Milan, Italy). Susceptibility to ertapenem, imipenem, meropenem and CZA was subsequently verified by broth microdilution using a Sensititre Gram Negative EURGNCOL plate (Thermo Fisher Scientific, Milan, Italy). All phenotypic results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints (The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters http://www.eucast.org.).

Sanger sequencing of *bla*KPC genes

The DNA of the 21 KPC-positive *K. pneumoniae* isolates was extracted with the DNeasy Blood&Tissue kit (Qiagen), according to manufacturer's instructions. The *bla*KPC gene was then amplified by PCR with the following primers: KPC-seq F: TGTCACTGTATCGCCGTC and KPC-seq R: CTCAGTGCTCTACAGAAAACC. PCR amplicons were purified using the kit Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, United States). DNA sequencing was performed using the Microsynth services (Microsynth Seqlab, Germany). The alignment was accomplished using ChromasPro software (Technelysium Pty Ltd, South Brisbane, Australia). The allelic variants were identified the BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Pulsed-Field Gel Electrophoresis (PFGE)

Pulsed-Field Gel Electrophoresis (PFGE) was carried out on all 21 *K. pneumoniae* isolates to investigate their genetic relatedness. The genomic DNA of each sample was digested with the *XbaI* restriction enzyme (45 U) and the genome fragments were separated on a CHEF mapper system (Bio-Rad Laboratories, Milan, Italy) at 14 °C at 6 V/cm for 20 h with an initial pulse time of 0.5 s and a final pulse time of 30 s. Lambda 48.5 kb concatamers (New England BioLabs, Beverly, MA, USA) were used as a molecular size marker. Dendrogram of strains relatedness was obtained with Fingerprinting II version 3.0 software (Bio-Rad Laboratories, Milan, Italy) using UPGMA, according to the criteria described by Tenover et al.[9] The dice correlation coefficient was used with a 1.0% of both position tolerance and optimization.

Detection of resistance genes with HybriSpot 12 system

On nine CZA-R KPC-producing *K. pneumoniae* strains (eight representative of different PFGE clones and subclones, and one strain not-typable by PFGE) the detection of resistance genes has been performed by the 56 target HybriSpot 12 PCR AUTO system (Eurospital Diagnostic, Trieste, Italy) with the MDR Direct Flow Chip kit, starting directly from bacterial colonies.

MultiLocus Sequence Typing (MLST)

Thirteen strains were chosen for MultiLocus Sequence typing (MLST) analysis based on the obtained pulsotypes, and one strain not-typable by PFGE was also added for this analysis, in order to cover all the strains involved in the outbreak. The MLST was performed according to the Pasteur Institute scheme (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html) by PCR and Sanger sequencing or *in silico* analysis. PCR amplicons from housekeeping genes were purified using a Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). Sequences were performed by the Microsynth Seqlab (Germany), and the analyses were performed with the DNA Sequencer software version 4.1.4.

Conjugation/transformation assay and cloning

The conjugal transfer of *bla*KPC-166 gene was tested for four strains from as many patients, chosen as representative of each ST. Conjugation was performed in liquid medium using *Escherichia coli* J62 strain (SM^R) as recipient. Transconjugants were selected on MacConkey agar plates (Scharlab, SL, Barcelona, Spain) containing streptomycin (1000 mg/L) (Sigma—Aldrich, St. Louis, MO, United States) and ampicillin (16 mg/L) (Sigma—Aldrich). Furthermore, transformation was carried out on 2_8 strain chosen as representative; plasmid extraction was performed using ZymoPURE Plasmid Miniprep Kit (Zymo research), and chemically competent *E. coli* Top10 cells were used as the recipient. Transformants were selected on Mueller-Hilton (MH) agar (Oxoid, Hampshire, UK) with 100 mg/L Ampicillin (Sigma—Aldrich). The *bla*KPC-166 gene from 2_8 was cloned into the pCR™2.1 vector by using TA cloning kit (Thermo Fisher Scientific) and primers *bla*KPC-166-CLO FW: 5'-CTAGCCTAAATGTGACAGTGGTTGT-3' and *bla*KPC-166-CLO RV: 5'-TTACATCCGGCCGCTACACCT-3'. The obtained vector was then expressed in chemically competent *E. coli* TOP10.

The presence of *bla*KPC-166 in the transconjugants/transformants and the typing of the plasmids were confirmed by PCR and PCR replicon typing (PBRT 2.0 kit), respectively. MICs values for MER and CZA were evaluated through gradient strips for donor, recipients. Moreover, MER and CZA MICs by gradient strips were investigated in KPC-2-producing *K. pneumoniae* 9_2023 and KPC-3-producing *K. pneumoniae* 98_2023, available in our Microbiology collection, comparing with MICs obtained by conjugation, transformation and TA cloning.

Whole-Genome Sequencing (WGS)

Ten representative strains were chosen for Whole-Genome Sequencing (WGS) based on the PFGE pulsotype (at least one representative for each pulsotype or subtype). The genomic DNA, extracted using the DNeasy Blood&Tissue kit (Qiagen), was sequenced on an Illumina HiSeq PE150 platform

(Illumina Inc., San Diego, CA, USA) with a 350-bp paired-end sequencing, after libraries preparation with Nextera XT library preparation kit. Read quality check was assessed with FastQC. Reads were assembled within the Shovill pipeline, discharging short contigs, with low coverage, or pure homopolymers.[10] Assembled sequences were annotated using Rapid Annotation using Subsystems Technology (RAST server).[11] Resistome, plasmid replicon content, mobile elements, MLST and plasmid MLST (pMLST) were determined throught uploading the assembled sequences to ResFinder 4.1 and CARD, PlasmidFinder, ISfinder, MLST and pMLST, respectively, available at the Center for Genomic Epidemiology website (https://www.genomicepidemiology.org/). Comparative genome alignment was conducted through SnapGene software (https://www.snapgene.com/).

Phylogenetic analysis

Phylogenetic relationship between WGS analysed isolates and global genomes were investigated. Phylogenetic trees were obtained using core genome, recombination and single nucleotide polymorphisms (SNPs) by parsnp v1.2 (https://github.com/marbl/parsnp/), available in the harvest suite and using a corresponding reference genome. Graphic illustration of the tree was built with the interactive tree of life (iTOL) (https://itol.embl.de/). For the construction of the SNPs-based phylogeny, 464 *K. pneumoniae* genomes were retrieved from NCBI assembly database, including complete and draft genomes, and 26 additional genomes of ST22 *K. pneumoniae* from the Pasteur *K. pneumoniae* species complex database. CP025140.1 was included as reference genome.

A sequence-based typing analysis on the 6813 *K. pneumoniae* MLST allelic profiles available at Pasteur Database (Kp MLST scheme; https://pubmlst.org/organisms/klebsiella-pneumoniae) was generated using the PHYLOViZ Online Tool (https://online.phyloviz.net/index).

The outbreak reconstruction was performed using P-DOR.[12] As a first step, the pipeline contextualizes the query genomes with the most similar ones retrieved from BV-BRC according to their genetic similarity. For each query genome, the 20 nearest ones were selected, for a total of 34 strains included as background. Once the background genomes are added to the query dataset, the core-SNPs phylogeny and SNP distance matrix are generated. Additionally, the metadata of hospitalized patients were included to allow P-DOR to reconstruct the contact network. Timeline of hospitalized patients was generated using "contact_network.R" script within the P-DOR software.

3D modelling

Molecular modelling for KPC-2 (WP_004199234.1), KPC-3 (WP__004152395.1), KPC-94 (WP_219804981.1) and KPC-166 was performed using the PHYRE2 Protein Fold Recognition Server (http://www.sbg.bio.ic.ac.uk/~phyre2/html/) and the resulted 3D structure images were visualized through EzMol — Molecular display wizard (http://www.sbg.bio.ic.ac.uk/ezmol/).

Data availability

The nucleotide sequence of *bla*KPC-166 has been uploaded to GenBank under the accession number OQ592369. The nucleotide sequences of the genomes were deposited and are available in GenBank under the BioProject number PRJNA1042821.

Results

Outbreak description

From October 2021 to March 2022, 16 inpatients admitted to the COVID-ICU of the ASST Grande Ospedale Metropolitano Niguarda, Milan (IT) were found to be colonized (N = 3) and/or infected (N = 14) by CZA-resistant (CZA-R) *K. pneumoniae* isolates. Demographic information and clinical course of patients are summarized in Supplementary Material S1.

Fourteen patients (88%) were hospitalized for SARS-CoV-2-related acute respiratory distress syndrome (ARDS), one for bacterial endocarditis and one for cardiac arrest. Diagnosis of colonization was made with a weekly surveillance swab (nasal, pharyngeal and rectal), and endotracheal aspiration culture. Ten patients hospitalized for ARDS (62.5%) received corticosteroids upon admission to the ICU, none received IL-6 inhibitors or other immunomodulating drugs. The timeline of the *outbreak* is represented in Fig. 1. Five patients (31%) were on antibiotic (levofloxacin, piperacillin/tazobactam plus vancomycin, ceftriaxone and ceftriaxone plus vancomycin, respectively) at admission to the ICU, mainly as empirical therapy (n = 4/5).

Thirteen patients (81%) had a bloodstream infection (BSI): 3/13 primary BSI, 2/13 catheter-associated, 5/13 secondary to pneumonia, 3/13 secondary to urinary tract infection. Three patients presented rectal, urinary, and respiratory colonization, respectively, but did not develop systemic infection. Targeted antimicrobial therapy was chosen on a case-by-case basis depending on the patient's clinical status and on the site of infection. Pneumonia-associated bloodstream infections were treated with meropenem/vaborbactam (MEV) (n = 3) or meropenem (MER) plus fosfomycin (n = 2), while primary, urinary tract-associated and catheter-related BSIs received carbapenem monotherapy (n = 4) or combination therapy (n = 4) depending on clinical severity. No recurrence was recorded, apart from a patient (patient ID 8) erroneously treated with CZA, which was successfully retreated with MEV. Three (19%) patients died in ICU, one (6%) patient died after ICU discharge. Only two (13%) patients (Patient ID 16 and 17 in Supplementary Material S1) had been treated with CZA before the first identification of CZA-R *K. pneumoniae*, though all patients had received at least one antimicrobial before the evidence of colonization or infection. Detailed data are reported in Supplementary Material S1.

Microbiological findings

A total of 21 distinct isolates were obtained from the 16 patients involved in the outbreak. The isolates were

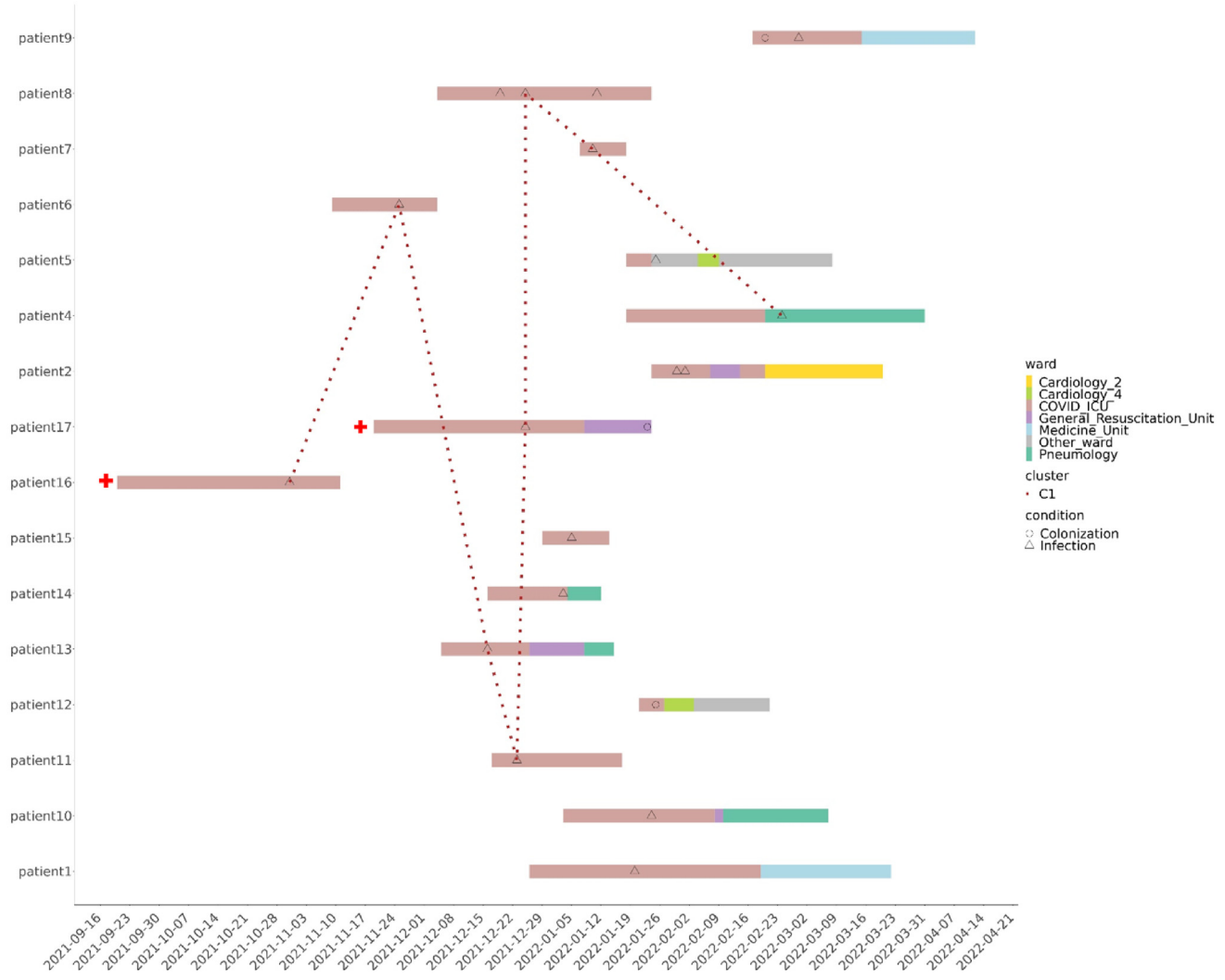

Figure 1. Timeline depicting the movements of the 16 hospitalized patients. The circles represent the bacterial strains isolated from infection (e.g. blood), the triangles bacterial strains isolated from colonization (rectal_swab). The three genomics samples belonging to ST307 are linked according to their SNP distance below the threshold of 21 SNPs. The red crosses indicate patients treated with CZA.

obtained from blood (n = 14), rectal swabs (n = 3), respiratory samples (n = 3), and urine (n = 1). The metadata of the *K. pneumoniae* isolates and antimicrobial susceptibility profiles are outlined in Supplementary Material S1. All the isolates were resistant to the extended-spectrum cephalosporins, CZA, ceftolozane/tazobactam but retained full carbapenem susceptibility. In detail, n = 19 isolates showed MER MICs even below the EUCAST epidemiological cut-off value ($\leq$ 0.125 μg/mL) and in two cases the MICs ranged from 0.25 to 1 μg/mL (Supplementary Material S1).

All CZA-R isolates were investigated for carbapenemases production, resulting positive for a KPC-type enzyme by both phenotypic and genotypic assay. Remarkably, they were unable to grow on the selective CHROMID CARBA medium, while they grew on CHROMID ESβL medium.

Genomic comparison and relatedness

All the strains were evaluated for their clonal relations by PFGE, that detected two pulsotypes: A in 18/21, B in 1/21 (Fig. 2). The clone A included five subclones (A1-A5). The remaining two strains, collected from the same patient, resulted untypable by PFGE.

MLST using Pasteur scheme was assessed for 14 strains, highlighting the predominance of the high-risk clone ST307 in 11 out of 14 cases. Moreover, four further STs have been detected in one strain each: ST22, and the newly assigned ST6342, ST6418 and ST6811. The ST6342, ST6418 and ST6811 are here described for the first time. The ST6342 and ST6811 represent a single locus variant of ST307. ST6342 presented two SNPs in *pgi* gene at positions 25 (T25C) and 71 (T71G), changing the allelic variant from 52

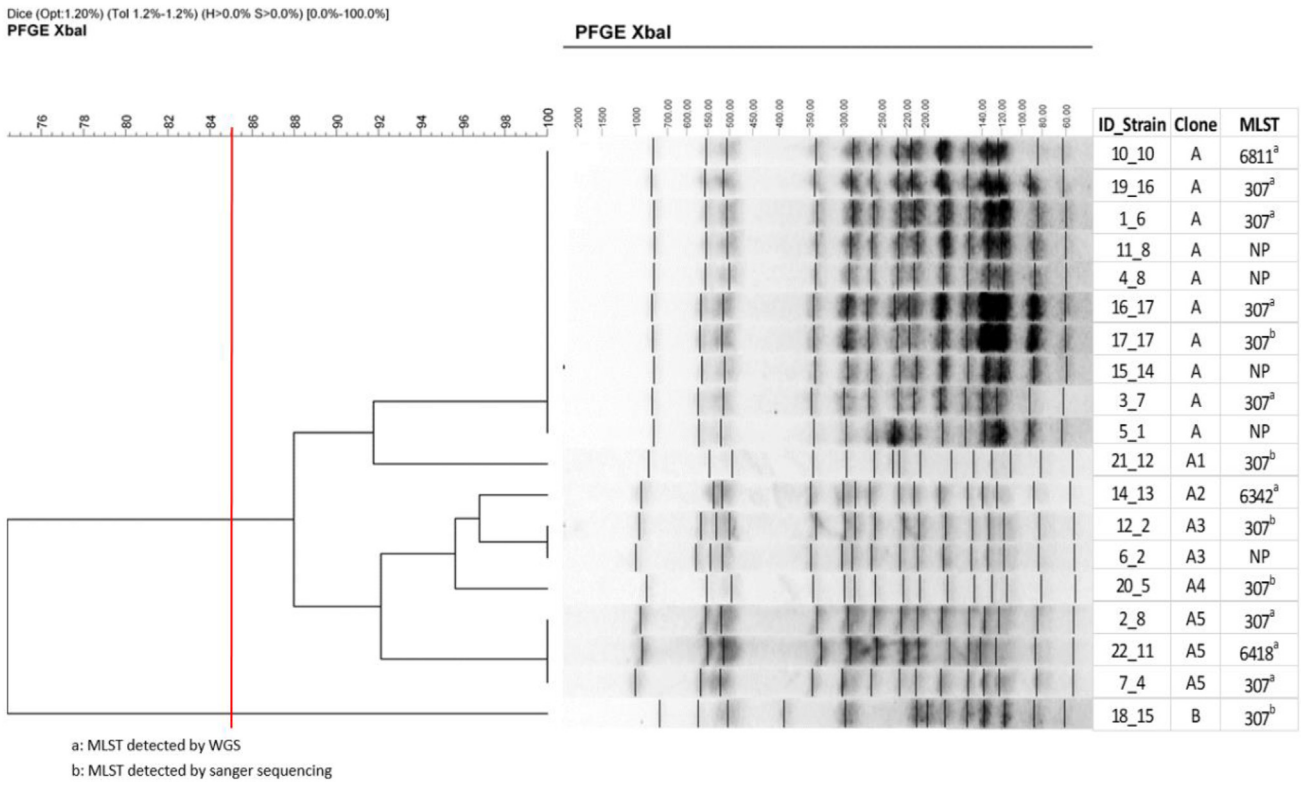

Figure 2. Cluster analysis of the 21 CZA-R *K. pneumoniae* strains. The scale bar at the top (left) indicates similarity coefficient (expressed in percentage). The identity threshold is set to 85%.

to 5. ST6811 presented one SNP in *tonB* gene at positions 233 (G233T), changing the allelic variant from 7 to 925. ST6418 presents two locus variants compared to ST307: in *mdh* gene, SNP T432C determines the change in *mdh* allelic 1 instead of 2, while in *pgi* gene the SNP T25C changes the *pgi* variant from 52 to 1. ST6342 and ST6811 showed high relation with the high-risk clone ST307, while ST6418 seemed to be originated from ST515, as suggested by the PHYLOVIZ analysis (Supplementary Fig. S1).

Considering genomic relatedness, the isolates related to ST307 of this study are closed to other deposited genomes presenting in some cases a *bla*KPC-type gene (*bla*KPC-2 and *bla*KPC-3) (Fig. 3A). The strict genetic relatedness within the Cluster C1 was highlighted by the maximum value n = 11 of SNPs, pointing out a clonal relation (Fig. 3B).

On the other hand, the strain 2_8, belonging to ST22, showed genomic relatedness with ESβL-producing *K. pneumoniae* strain isolated in Norway (Pasteur: DAFXJB01.1, GeneBank: GCA_021924005.1), and with a VIM-producing *K. pneumoniae* from Greece (Pasteur: DAGQCA01.1, GeneBank: GCA_022268335.1) (Fig. 4).

Molecular investigations

PCR investigation highlighted the presence of *bla*KPC-type determinant in all the studied isolates. WGS, conducted on 10 strains, revealed an identical resistome content: *aac(3)-IIa*, *aac(6')-Ib-cr*, *qnrB1*, *catB4*, *tet(A)*, *dfrA14*, *bla*OXA-1, *bla*CTX-M-15 and a *bla*KPC-94 with a single nucleotide substitution in position 811 (T811C). In addition, three out of four strains carried *bla*SHV-28 and substitutions in GyrA-83I and ParC-80I. Moreover, one strain harbored *strA, strB*, *bla*TEM-1, *bla*SHV-1 but lacked of substitutions in GyrA and ParC (Table 1). At the chromosomal level, mutations that potentially contribute to increased resistance to third generation cephalosporins and carbapenems were detected in OmpK36 (N49S, L59V and T184P) and in OmpK37 (I70M, I128M, N230G and M233_None234insQ) for all the ten WGS isolates.

Description of a novel *bla*KPC variant

The Sanger sequencing and the BLAST results on *bla*KPC showed the highest identity with *bla*KPC-94 variant (NG_076680.1) (identity 99.89% and query 100%). Comparing with *bla*KPC-94, the novel *bla*KPC differed for a single nucleotide substitution in position 811 (T811C), which leaded to a change in the amino acidic sequence, replacing a residue of Tyrosine (Y) with Hystidine (H) in position 271 (Y271H) (Fig. 5, Supplementary Fig. S3). This mutation was detected in all the 21 isolates. The new KPC variant (originally named *bla*KPC-94*T811C) has been uploaded in GenBank database and assigned as *bla*KPC-166 (Accession number OQ592369). KPC-166 owned conserved α (1—12) and β (1—6) loops, while the Ω loop, involved in the binding and catalytic activity, is located in position 164—179 aa (Fig. 5). The KPC-166 showed high level of identity with KPC-94 (WP_219804981.1) (99.66.% identity and 100%query), KPC-2 (WP_004199234.1) (99.32% identity and 100% query) and with KPC-3 (WP_004152395.1) (98.98%

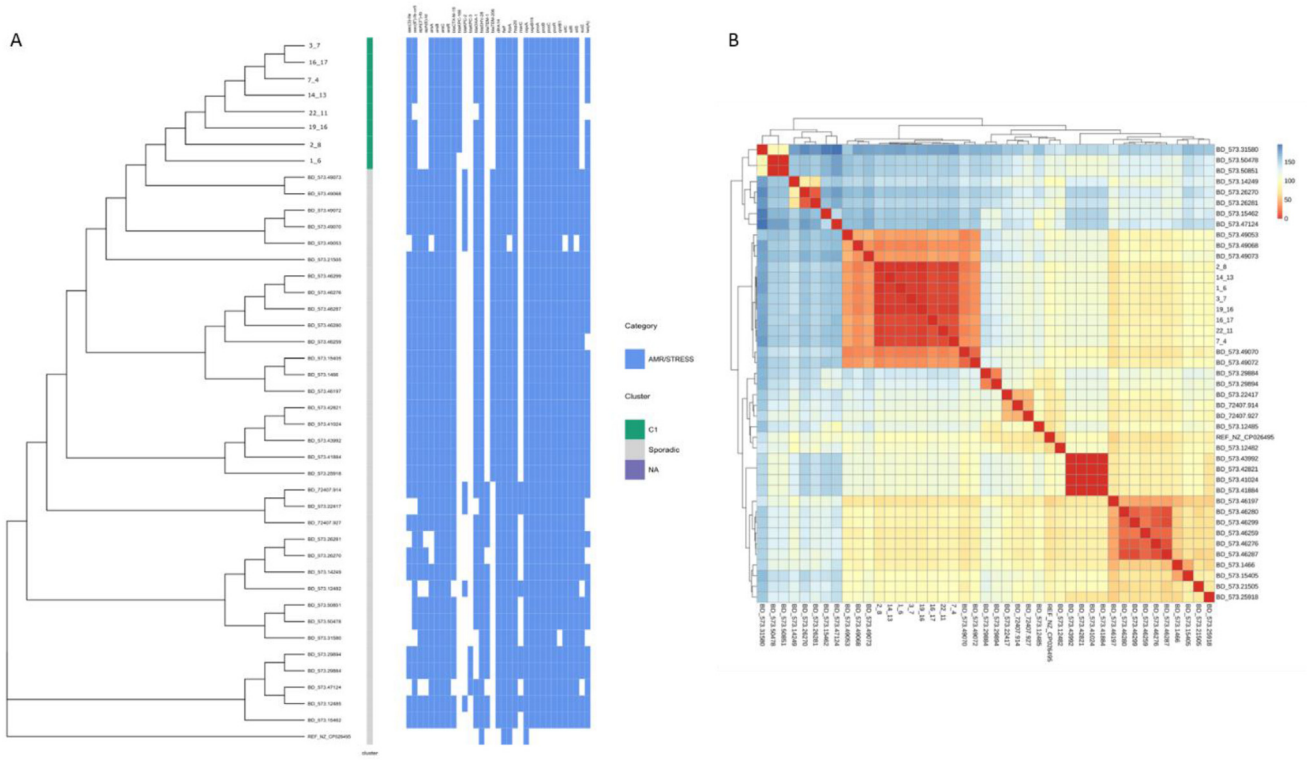

Figure 3. A: SNPs-based phylogeny of the 307-related strains. B: heatmap of the SNPs number detected.

identity and 100% query). Compared with KPC-2 and KPC-3, both KPC-166 and KPC-94 lacked the Leucine (L) residue in position 168 and carry the substitution N169H (Fig. 5, Supplementary Fig. S3). These two mutations fall in the Ω loop region of both KPC-94 and KPC-166, explaining in both cases the CZA resistance and the MER susceptibility (Fig. 5, Supplementary Fig. S3). Moreover, based on the prediction analysis of protein structure, the modification Y271H occurred in the helix α12 (270-287aa), which partly constitutes the loop 266—275, a secondary mutational hot spots associated with an increased resistance to CAZ (Fig. 5).[13]

Genetic context of the *bla*KPC-166 and plasmid characterization

Based on *in silico* analysis, *bla*KPC-166 is located on pKpQIL plasmid, that belongs to IncFII$_{k2}$ group. *bla*KPC-166 is inserted in the composite Tn4401*a* transposon. The entire mobile unit is flanked by a *insA* in upper position and by the *Tn3* of ISPsy*42*. The entire mobile unit shared 99.96% identity with *Tn4401*a-harboring *bla*KPC-2 (KR052098.1) and *bla*KPC-94 (GCF_019430835.1).[14] All the strains contained the replicon FIB KQ belonging to IncFII$_{k2}$.

Horizontal transferability and cloning of *bla*KPC-166

The aforementioned plasmid resulted horizontally transferable by both conjugation and transformation (Table 2). Moreover, *bla*KPC-166 was cloned in a pCR2.1 vector and expressed in chemically competent Top10 cells, showing resistance to CZA (MIC = 4 μg/mL) and retaining susceptibility to MER (MIC = 0.016 μg/mL) (Table 2).

Discussion

In the current study we described an outbreak caused by CZA-R *K. pneumoniae* strains in a COVID-Intensive Care Unit in Northern Italy. We investigated the molecular mechanisms underlying the CZA resistance observed, and we identified a novel variant of *bla*KPC, here named *bla*KPC-166.

CZA is a valid option against CRE infections but, as described elsewhere, reports concerning CRE showing CZA resistance are rapidly increasing.[4] *bla*KPC mutations, occurring after CZA treatment, are pointed as leading cause of CZA resistance phenotypes.[15] The most common substitutions occur in the Ω loop structure, as L169P, A177E and D179Y.[4] KPC-166 showed high identity with KPC-94, sharing the same Ω loop sequence. KPC-94 was only described in a clinical case of ST512 *K. pneumoniae* from Spain and, similarly to KPC-166, it is able to confer resistance to CZA; however, the carbapenem hydrolyzing activity in both KPC-94 and KPC-166 results compromised.[14] KPC-2 and KPC-166 shared the same histidine residue in the helix α12 at position 272, while the Ω loop sequence resulted altered.

The high-risk clone ST307 emerged in the mid-1990s and rapidly spread in all continents except Antartica; it is endemically disseminated in Colombia, Italy, South Africa and USA,[16] with consequences in the dissemination of

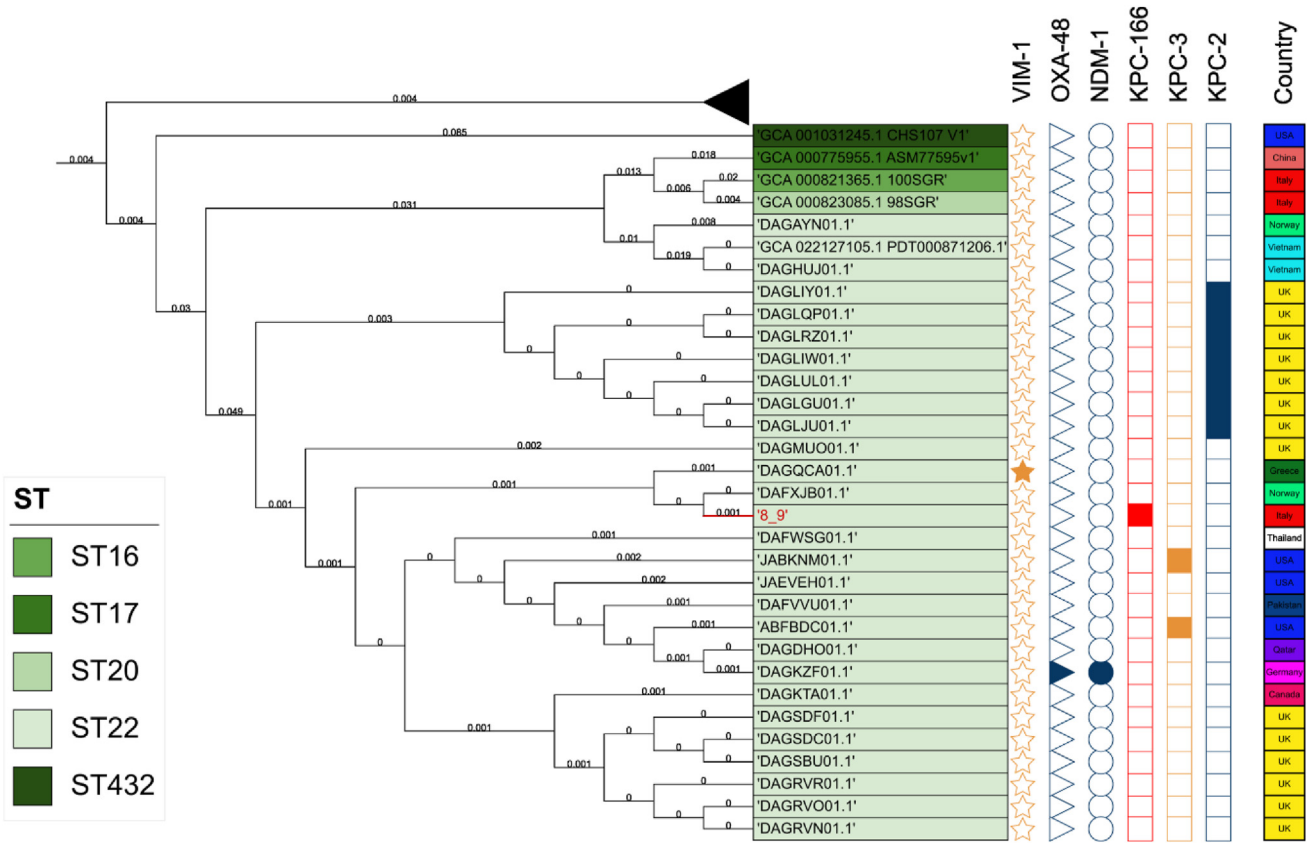

Figure 4. SNPs-based phylogeny of the ST22 *K. pneumoniae* isolates with 490 genomes downloaded from the NCBI database. The analysis was conducted using parsnp v1.2 and visualized with iTOL v6. Red nodes indicate the ST22 isolates harboring the KPC-166 variant. Filled shape = presence of the enzyme; empty shape = absence of the enzyme. Dark blue square: KPC-2; orange square: KPC-3; red square: KPC-166; dark blue dot: NDM-1; dark blue triangle: OXA-48; orange star: VIM-1. Gray triangle indicates collapsed nodes. The right colored boxes show the countries of collection.

various clinically relevant ESβLs, as CTX-M-15,[17] and carbapenemases, as KPC-2 and KPC-3.[18] The *in vivo* emergence of CZA resistance has been already reported in the ST307 *K. pneumoniae*.

Regarding the three new STs: ST6342 and ST6811 are single locus variants of ST307, in *pgi* (5) and *tonB* (925), respectively. The remaining ST6418 showed two locus variants compared to ST307: in *mdh* (1) and *pgi* (1). Interestingly, this latter ST seemed to be related with ST515, a member of the CG15, known to be successful clone, causing outbreaks of CTX-M-15 and/or carbapenemase producers worldwide.[19]

ST22 is an unusual clone described in clinical cases from Mexico and China.[20,21] In the available reports, ST22 is associated to the occurrence of *bla*NDM-like genes on IncF-type plasmids.[20] Recently, the co-presence of *bla*NDM-1 and *bla*KPC-2 on IncFII plasmids has been described in pan-drug ST22 *K. pneumoniae* strains, causing fatal sepsis events in China.[21] The presence of highly conjugative plasmids carrying a new KPC variant in high-risk and emerging clones, could represent further challenges to face in clinical settings.

As previously suggested by pharmacokinetic/pharmacodynamic analysis, the emergence of CZA-resistance could have been favored by suboptimal exposure to CZA.[22] Considering the development and the evolution of the COVID-ICU outbreak (Fig. 1), inadequate CZA exposure — especially in ventilation-associated pneumonia — could have had a role in the emergence of the first strain. Currently, there is no evidence regarding the most effective treatment regimen for these strains, which harbor carbapenemase genes but retain susceptibility to carbapenems. Moreover, these strains are more likely emerging in patients with a previous history of CRE colonization/infection, and persisting as minor population. Previously reported cases of infections sustained by CZA-R carbapenem-susceptible *Enterobacterales* had been treated with carbapenem monotherapy or combinations (either with aminoglycosides, colistin or tigecycline), though the reported overall mortality of 50% is significantly higher compared to that observed in ESβL-producing *Enterobacterales*.[23] Moreover, both *in vitro* and *in vivo* selection of carbapenem-resistance in CZA-R carbapenem-susceptible *Enterobacterales* strains has been described.[24,25] Differently, treatment with MEV has been proposed as rescue therapy especially in critically ill immunocompromised patients.[26] Since the restoration of MER resistance has been described in CZA-R *K. pneumoniae*, MEV or MER-based combination therapy might prevent phenotype reversion with subsequent therapeutic failure in deep-seated

Table 1 Molecular results of the 21 *K. pneumoniae* strains. In bold are indicated the strains that underwent WGS.

ID strain	Isolation date	PFGE clone	MLST	KPC variant	HybriSpot results	WGS results
19_16	31/10/2021	A	307	KPC-166	*bla*SHV; *bla*CTX-M; *bla*KPC; *aac(6')-Ib; qnrB; oqxA; oqxB; catB3*	*aac(3)-IIa, aac(6')-Ib-cr, qnrB, catB3, tetA, dfrA14, bla*OXA-1, *bla*CTX-M-15, *bla*SHV-28, *acrR* (P161R, G164A, F172S, R173G, L195V, F197I and K201M); OmpK36 (N49S, L59V and T184P); OmpK37 (I70M, I128M, N230G and M233_None234insQ)
1_6	26/11/2021	A	307	KPC-166	*bla*SHV; *bla*CTX-M; *bla*KPC; *aac(6')-Ib; qnrB; oqxA; oqxB*	*aac(3)-IIa, aac(6')-Ib-cr, qnrB, catB3, tetA, dfrA14, bla*OXA-1, *bla*CTX-M-15, *bla*SHV-28, *acrR* (P161R, G164A, F172S, R173G, L195V, F197I and K201M); OmpK36 (N49S, L59V and T184P); OmpK37 (I70M, I128M, N230G and M233_None234insQ)
11_8	20/12/2021	A	NP**	KPC-166	NP**	NP**
16_17	26/12/2021	A	307	KPC-166	NP**	*aac(3)-IIa, aac(6')-Ib-cr, qnrB, catB4, tetA, dfrA14, bla*OXA-1, *bla*CTX-M-15, *bla*SHV-28, *GyrA*-83I;*ParC*-80I; OmpK36 (N49S, L59V and T184P); OmpK37 (I70M, I128M, N230G and M233_None234insQ)
15_14	04/01/2022	A	NP**	KPC-166	NP**	NP**
3_7	11/01/2022	A	307	KPC-166	NP**	*aac(3)-IIa; aac(6')-Ib-cr, qnrB, dfrA14, bla*CTX-M-15, *bla*SHV-28, *GyrA-83I;ParC-80I; acrR* (P161R, G164A, F172S, R173G, L195V, F197I and K201M); OmpK36 (N49S, L59V and T184P); OmpK37 (I70M, I128M, N230G and M233_None234insQ)
4_8	12/01/2022	A	NP**	KPC-166	NP**	NP**
5_1	21/01/2022	A	NP**	KPC-166	NP**	NP**
17_17	24/01/2022	A	307	KPC-166	NP**	NP**
10_10	25/01/2022	A	6811	KPC-166	NP**	*aac(3)-IIa, aac(6')-Ib-cr, qnrB, catB3, tetA, dfrA14, bla*OXA-1, *bla*CTX-M-15, *bla*SHV-28, *acrR* (P161R, G164A, F172S, R173G, L195V, F197I and K201M); OmpK36 (N49S, L59V and T184P); OmpK37 (I70M, I128M, N230G and M233_None234insQ)
21_12	26/01/2022	A1	307	KPC-166	*bla*SHV; *bla*CTX-M; *bla*KPC; *aac(6')-Ib; qnrB; oqxA; oqxB*	NP**
14_13	17/12/2021	A2	6342	KPC-166	*bla*SHV, *bla*CTX-M, *bla*KPC, *aac(6')-Ib, qnrB, oqxA, oqxB, catB3*	*aac(3)-IIa, aac(6')-Ib-cr, qnrB, catB3, tetA, dfrA14, bla*OXA-1, *bla*CTX-M-15, *bla*SHV-28, *acrR* (P161R, G164A, F172S, R173G, L195V, F197I and K201M); OmpK36 (N49S, L59V and

(*continued on next page*)

Table 1 (*continued*)

ID strain	Isolation date	PFGE clone	MLST	KPC variant	HybriSpot results	WGS results
						T184P); OmpK37 (I70M, I128M, N230G and M233_None234insQ)
12_2	31/01/2022	A3	307	KPC-166	*bla*SHV, *bla*CTX-M, *bla*KPC, *aac(6')-Ib*, *qnrB*, *oqxA*, *oqxB*, *catB3*	NP**
6_2	02/02/2022	A3	NP**	KPC-166	NP	NP**
20_5	26/01/2022	A4	307	KPC-166	*bla*SHV, *bla*CTX-M, *bla*KPC, *aac(6')-Ib*, *qnrB*, *oqxA*, *oqxB*, *catB3*	NP**
22_11	24/12/2021	A5	6418	KPC-166	*bla*SHV; *bla*CTX-M; *bla*KPC; *qnrB; oqxA; oqxB*[a]	*aac(3)-IIa; qnrB, catB4, tetA, dfrA14, bla*OXA-1, *bla*CTX-M-15, *bla*SHV-28, GyrA-83I; ParC-80I; OmpK36 (N49S, L59V and T184P); OmpK37 (I70M, I128M, N230G and M233_None234insQ)
2_8	26/12/2021	A5	307	KPC-166	NP**	*aac(3)-IIa; aac(6')-Ib-cr, qnrB, catB4, tetA, dfrA14, bla*OXA-1, *bla*CTX-M-15, *bla*SHV-28, GyrA-83I; ParC-80I; OmpK36 (N49S, L59V and T184P); OmpK37 (I70M, I128M, N230G and M233_None234insQ)[b]
7_4	25/02/2022	A5	307	KPC-166	NP**	*aac(3)-IIa, aac(6')-Ib-cr, qnrB, catB3, tetA, dfrA14, bla*OXA-1, *bla*CTX-M-15, *bla*SHV-28, *acrR* (P161R, G164A, F172S, R173G, L195V, F197I and K201M); OmpK36 (N49S, L59V and T184P); OmpK37 (I70M, I128M, N230G and M233_None234insQ)
18_15	06/01/2022	B	307	KPC-166	*bla*SHV; *bla*CTX-M; *bla*KPC; *sul2; aac(6')-Ib; qnrB; oqxA; oqxB; catB3*	NP**
13_9	21/02/2022	NT*	NP**	KPC-166	*bla*SHV; *bla*CTX-M; *bla*KPC; *sul2*; *aac(6')-Ib*; *qnrB*; *oqxA*; *oqxB*	NP**
8_9	01/03/2022	NT*	22	KPC-166	NP**	*aac(3)-IIa, aac(6')-Ib-cr, strA, strB, qnrB, catB4, tetA, dfrA14, bla*OXA-1, *bla*TEM-1D, *bla*CTX-M-15, *bla*SHV-1, *sul2*; OmpK36 (N49S, L59V and T184P); OmpK37 (I70M, I128M, N230G and M233_None234insQ)[b]

* NT: Not typable.
** NP: Not performed.

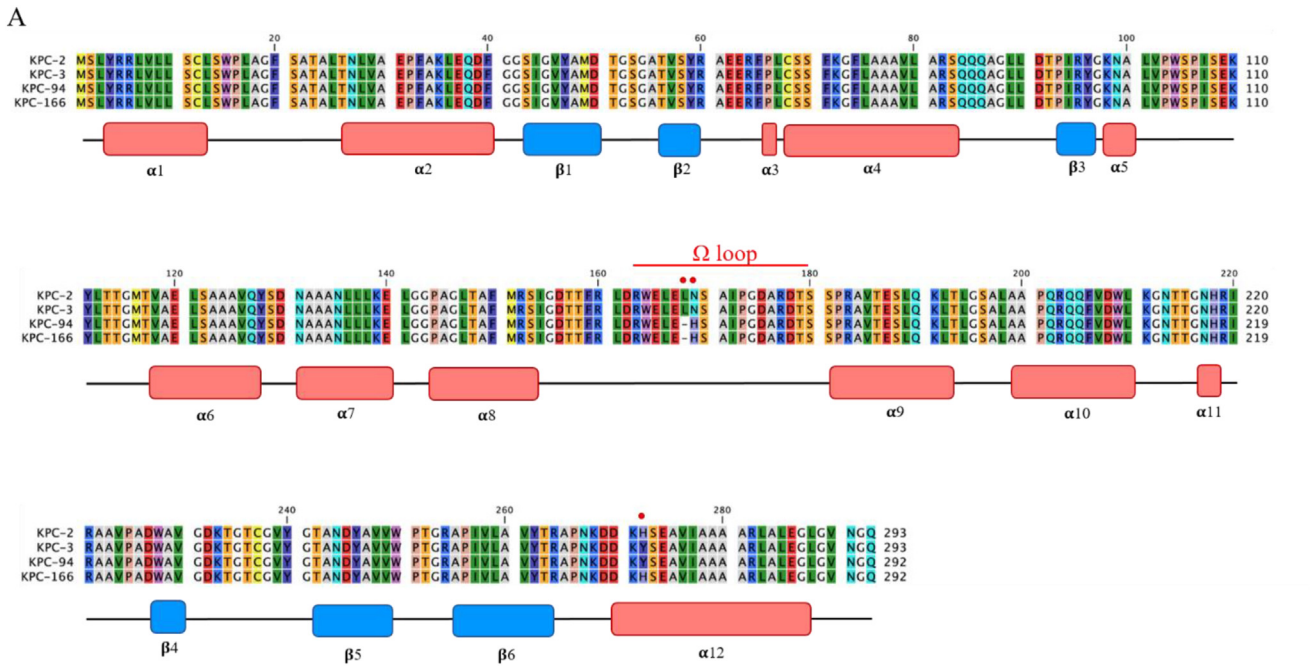

Figure 5. A: The KPC-166 protein (WP_279240783.1) in comparison with the prototype KPC-2 (WP_004199234.1) KPC-3 (WP_004152396.1) and KPC-94 (WP_219804981.1). Top, alignment of the four amino acidic sequences. Bottom, predicted secondary structure of KPC-166 indicating α-helices (α1 = 4-12aa; α2 = 26-40aa; α3 = 66aa; α4 = 68-83aa; α5 = 98-100aa; α6 = 118-127aa; α7 = 131-140aa; α8 = 144-153aa; α9 = 181-192aa; α10 = 199-210aa; α11 = 217-218aa; α12 = 270-287aa) in pink and β-sheets (β1 = 43-50aa; β2 = 56-59aa; β3 = 93-96aa; β4 = 228-230aa; β5 = 242-249aa; β6 = 255-262aa) in light blue. Red dots indicate mutations in the amino acidic sequence. The red line shows the Ω loop (164-179aa).

infections as pneumonia or intra-abdominal infections where prompt source-control is not a feasible option.

Tn*4401* is a Tn*3*-like transposon that has been largely associated to the spread of KPC-like carbapenemases.[27] The promiscuous trasposon Tn*4401*, of approximately 10 kb in length, is composed of a transposase gene (*tnpA*), a resolvase gene (*tnpR*), the *bla*KPC gene, and the insertion sequences IS*Kpn6* and IS*Kpn7*.[2] Tn*4401* has been integrated in different conjugative plasmids, contributing to the spread of KPC-like carbapenemases.[2] The classification of Tn*4401* includes five isoforms (Tn*4401a* – Tn*4401e*) which differs by deletion, at different extent, of the region upstream the *bla*KPC gene.[2] Tn*4401a* has been largely documented as vehicle in the spread of *bla*KPC-2 and *bla*KPC-3 among *Enterobacterales*.[2,28] Plasmids carrying Tn*4401a* found a highly compatible host in *K. pneumoniae* ST258.[29] *bla*KPC-166 was inserted in a Tn*4401a* transposon, which was located on a conjugative pKpQIL plasmid. pKpQIL is a self-conjugative plasmid belonging to IncFIIk$_2$ replicon, firstly described as KPC-vehicle in Israel.[2] Several studies have highlighted the major role of pKpQIL-like plasmids in the spread of KPC-like enzymes in Italy, Greece and USA.[30,31] Our findings revealed *i*) the ability of Tn*4401a* to harbour new KPC variants, as KPC-166, and *ii*) the

Table 2 MIC values (in μg/ml) for MER and CZA obtained during conjugation, transformation and TA cloning.

ID strain	Species	KPC-type	MIC MER	MIC CZA	Reference
E. coli J62	*E. coli*	ND[a]	≤0.12	0.016	This study
2_8	*K. pneumoniae*	KPC-166	≤0.12	>8	This study
E. coli J62*2_8	*E. coli*	KPC-166	0.016	4	This study
22_11	*K. pneumoniae*	KPC-166	≤0.12	>8	This study
E. coli J62*22_11	*E. coli*	KPC-166	0.047	4	This study
8_9	*K. pneumoniae*	KPC-166	≤0.12	>8	This study
E. coli J62*8_9	*E. coli*	KPC-166	0.047	4	This study
Top10	*E. coli*	ND	≤0.12	0.016	This study
Top10*2_8	*E. coli*	KPC-166	0.016	4	This study
Top10*KPC-166-pCR2.1	*E. coli*	KPC-166	0.016	4	This study
9_2023	*K. pneumoniae*	KPC-2	1.5	2	This study
98_2023	*K. pneumoniae*	KPC-3	>32	1	This study
PAC2_B	*K. pneumoniae*	KPC-94	2	>16/4	14

[a] ND: Not detected.

successful integration of such transposon in the highly conjugative pKpQIL plasmid. This combination has the potential to acquire and, consequently, disseminate new KPC variants with reduced affinity to CZA, leading to an increase in CZA-R cases also in hosts not previously exposed to CZA.

The presence of low MIC values in carbapenemase-producing strains poses a significant challenge for both surveillance and clinical management. In the case here described, MER susceptibility was fully restored in all but one isolates, resulting in an inability to grow on selective plates and in a phenotype easily associable to ESβL-producer. A similar issue has been described in few cases in Italy; in another COVID-19 ICU, where 21 patients were colonized by KPC-33-producing and CZA-R *K. pneumoniae*, with only two patients previously exposed to CZA,[32] and in a case of a KPC-33-producing ST512 *K. pneumoniae* from a surveillance rectal swab at the Perugia University hospital.[33] In another report, among four cases of CZA-R KPC-3 *K. pneumoniae* only one exhibited a wild-type MIC for MER.[34] A more recent study described the *in vivo* emergence of KPC-producing and CZA-R *K. pneumoniae* with a complete restoration of MER susceptibility.[14]

Based on the literature and our results, a culture-based approach turns out to be insufficient for the identification of variants involved in CZA resistance. This kind of CZA-R strains prompt further investigation in order to apply adequate infection control measures and to select the appropriate antibacterial therapy. Thus, the aid of molecular-based approach results crucial for the detection of such strains in clinical settings. However, even though the use of PCR-based rapid diagnostic tests was found to increase the rate of early and appropriate therapy for bloodstream infections by CRE,[35] molecular-based approaches alone are not enough for the identification of unusual resistance profiles and the use of phenotypic AST remain crucial in selecting the proper treatment, especially in patients with previous exposure to antimicrobials and a long hospital stay.

Three were the noteworthy characteristics of this variant: the resistance to CZA, the MICs for carbapenems far below the ECOFF and the inability to grow on selective media.

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Declaration of competing interest

No conflict of interest is declared by any of the Authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2024.03.004.