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Tracking Nosocomial *Klebsiella pneumoniae* Infections and Outbreaks by Whole-Genome Analysis: Small-Scale Italian Scenario within a Single Hospital

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Multidrug-resistant (MDR) *Klebsiella pneumoniae* is one of the most important causes of nosocomial infections worldwide. After the spread of strains resistant to beta-lactams at the end of the previous century, the diffusion of isolates resistant to carbapenems and colistin is now reducing treatment options and the containment of infections. Carbapenem-resistant *K. pneumoniae* strains have spread rapidly among Italian hospitals, with four subclades of pandemic clonal group 258 (CG258). Here we show that a single Italian hospital has been invaded by three of these subclades within 27 months, thus replicating on a small scale the "Italian scenario." We identified a single clone responsible for an epidemic outbreak involving seven patients, and we reconstructed its star-like pattern of diffusion within the intensive care unit. This epidemiological picture was obtained through phylogenomic analysis of 16 carbapenem-resistant *K. pneumoniae* isolates collected in the hospital during a 27-month period, which were added to a database of 319 genomes representing the available global diversity of *K. pneumoniae* strains. Phenotypic and molecular assays did not reveal virulence or resistance determinants specific for the outbreak isolates. Other factors, rather than selective advantages, might have caused the outbreak. Finally, analyses allowed us to identify a major subclade of CG258 composed of strains bearing the yersiniabactin virulence factor. Our work demonstrates how the use of combined phenotypic, molecular, and whole-genome sequencing techniques can help to identify quickly and to characterize accurately the spread of MDR pathogens.

Klebsiella pneumoniae is a major nosocomial pathogen that is rapidly spreading in hospitals worldwide, mainly due to the common occurrence of multidrug-resistant (MDR) strains (1). Infections caused by this pathogen are difficult to eradicate, since *K. pneumoniae* carries genes for resistance to the majority of antimicrobial drugs, including carbapenems (2, 3). The first strain of carbapenem-resistant *K. pneumoniae* was isolated in 1996; the plasmid-encoded determinant was named *K. pneumoniae* carbapenemase (KPC) and was indicated as the bla_{KPC} gene (4). Since then, KPC-producing *K. pneumoniae* strains have been spreading worldwide. Additional carbapenemases (bla_{NDM}, bla_{OXA-48}, bla_{VIM}, and bla_{IMP-1}) have now been reported for MDR *Enterobacteriaceae*, including *K. pneumoniae* (5–8). A last-resort treatment for infections caused by MDR Gram-negative bacteria is represented by membrane-acting polymyxins such as colistin, but resistance to this antibiotic in *K. pneumoniae* is also emerging (9, 10).

In addition to the study of genes providing resistance to antibiotics, genetic factors involved in the variable levels of virulence of different isolates of *K. pneumoniae* are currently highly investigated but only partially understood. Among the most important virulence factors are fimbrial genes (*mrk* and *fim* operons), which mediate adherence to surfaces and host tissues (11, 12). Another important aspect involved in the colonization of the host is the presence of genes for iron uptake systems such as aerobactin (13), enterobactin (*ent* operon) (14), and yersiniabactin (*irp* and *ybt* genes) (15). Capsular types, particularly K1 and K2, and hypermucoviscosity, favored by the positive regulator genes *rmpA* and *rmpA2*, are also important for *K. pneumoniae* virulence. Capsule production increases resistance to phagocytosis and other immune response components (16, 17). For detailed descriptions of these and other potential virulence factors of *K. pneumoniae*, see references 18 and 19.

Most of the KPC-producing *K. pneumoniae* strains isolated worldwide have been attributed to clonal group 258 (CG258) (19, 20). Recent phylogenomic analyses showed that four different subclades of pandemic CG258 are present in Italy, indicating entrance into the country on at least four different occasions during the period of 2008 to 2010 (21). The spread of MDR *K. pneumoniae* in hospitals and nursing homes in Italy is known to have occurred very rapidly, with a diffusion pattern that has been de-

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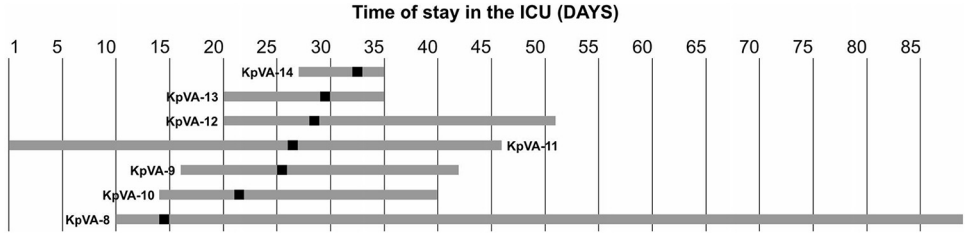

FIG 1 Time frames of stays in the ICU for the seven patients involved in the *K. pneumoniae* outbreak. Horizontal bars, length of stay for each patient. Black squares, day of the first isolation of *K. pneumoniae* for each patient.

scribed as the "Italian scenario" (22). The worldwide spread of *K. pneumoniae* is due, in part, to failures in the early identification of MDR strains, as well as high rates of recombination and horizontal gene transfer (21, 23, 24).

Whole-genome sequencing is now offering the possibility of in-depth characterization of bacterial isolates, and it holds the potential to reconstruct the origin and diffusion of nosocomial infections and outbreaks (19, 25). Here we present a phylogenomic study of 16 isolates from a single hospital in northwestern Italy that were collected between 2011 and 2013, including an epidemic outbreak in 2013 that involved seven patients. Genomes from these isolates were compared with 319 publicly available genomes, representing the available global genomic diversity of *K. pneumoniae*. Phylogenomic analysis, together with phenotyping assays and molecular characterization of drug resistance determinants and virulence genes, allowed us to trace the origins of sporadic infections and the outbreak, to describe the monophyletic origin of a yersiniabactin-positive subclade of CG258, and to detect a common genetic trait in colistin-resistant strains.

MATERIALS AND METHODS

Nosocomial infections with *K. pneumoniae* and hospital outbreak. Between January 2011 and March 2013, 16 cases of infection due to carbapenem-resistant *K. pneumoniae* occurred at the Ospedale di Circolo e Fondazione Macchi (Varese, Italy). Seven cases that occurred in the intensive care unit (ICU) during a short period were part of a single epidemic event that started in February 2013 (Fig. 1). Evidence indicated that a 69-year-old man was patient zero (indicated as KpVA-8 in Fig. 1). He had been transferred to the ICU from a nearby hospital, with an already diagnosed infection due to KPC-producing *K. pneumoniae*. During his stay in the ICU, infection spread to six other patients.

Bacterial isolates. A total of 16 nonduplicated isolates of *K. pneumoniae* were investigated, specifically, the first isolate obtained from each patient. Multiple *K. pneumoniae* isolates were obtained subsequently from each patient, for clinical reasons (e.g., spread of infection to novel body sites) or in the course of surveillance studies. Clinical specimens included urine, blood, bronchoalveolar lavage fluid, sputum, tracheal aspirate, and wound specimens. During the outbreak period, ICU patients were screened every 3 days for surveillance, using nasal, armpit, inguinal, and rectal swabs. Species identification and antibiotic susceptibility tests were performed with the FDA-approved Phoenix automated microbiology system (Becton, Dickinson, Sparks, MD). Additional quantitative assays were performed using Etest strips (bioMérieux, Marcy l'Etoile, France) on Mueller-Hinton agar II plates (Becton, Dickinson), according to clinical breakpoints from the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Short descriptions of the investigated isolates are presented in Table 1, while Etest MICs are reported in Table 2.

Direct sequencing of 16S rRNA, drug resistance genes, and virulence factors. Starting from pure cultures on Mueller-Hinton agar, bacterial DNA was obtained by lysozyme pretreatment (Sigma-Aldrich, Milan, Italy) followed by extraction with a QIAmp DNA Blood minikit (Qiagen, Milan, Italy). Confirmatory identification was performed via direct sequencing of the 16S rRNA gene. PCR was performed using AmpliTaq Gold with buffer I (Applied Biosystems, Life Technologies, Monza, Italy) in 50-μl mixtures, according to the manufacturer's instructions. PCR primers were synthesized by Sigma-Genosys (Haverhill, United Kingdom). Published primers and thermal protocols were used (26). DNA fragments were analyzed by electrophoresis on a 1.5% agarose gel in TBE buffer (89 mM Tris-borate and 2 mM EDTA [pH 8.3]) containing GelRed (10,000× in water; Biotium, DBA Italy, Segrate, Italy). PCR products were purified and sequenced on an ABI Prism 310 sequencer (Life Technologies). Sequences were compared with those in GenBank.

PCR assays for detecting antimicrobial resistance genes (27) and virulence factors were performed according to published protocols. Genes coding for adhesion fimbriae, enterobactin, and yersiniabactin siderophores were searched for, as follows: *fimH* gene, coding for type 1 fimbriae (28); *mrkA* gene, coding for the major subunit protein, and *mrkD* gene, coding for the adhesin, for type 3 fimbriae (29, 30); e*ntE* gene, coding for synthase subunit E, and e*ntB* gene, coding for isochorismatase, for enterobactin siderophore synthesis (14); y*btS* gene, coding for salicylate synthase, for yersiniabactin siderophore synthesis; *irp-1* and *irp-2* genes, related to yersiniabactin siderophore (15). Direct sequencing was performed as reported above.

Whole-genome sequencing and assembly. Whole-genome DNA was sequenced using an Illumina MiSeq platform (Illumina Inc., San Diego, CA), with a paired-end run of 2 by 250 bp, after Nextera XT paired-end library preparation. Sequencing reads were assembled using MIRA 4.0 software (31) with accurate *de novo* settings.

TABLE 1 Clinical isolates and main properties

Patient no.	Clinical isolate status	Date of isolation (mo/day/yr)	Source[a]	Sequence type
KpVA-4	Sporadic	1/11/11	B	ST258
KpVA-5	Sporadic	1/28/11	B	ST258
KpVA-6	Sporadic	3/14/11	B	ST258
KpVA-7	Sporadic	5/3/11	B	ST258
KpVA-1	Sporadic	10/31/12	SP	ST512
KpVA-2	Sporadic	1/30/13	BAL	ST258
KpVA-8	Epidemic	2/1/13	BAL	ST512
KpA-10	Epidemic	2/8/13	BAL	ST512
KpVA-9	Epidemic	2/12/13	BAL	ST512
KpVA-11	Epidemic	2/13/13	B	ST512
KpVA-12	Epidemic	2/15/13	TA	ST512
KpVA-13	Epidemic	2/16/13	WS	ST512
KpVA-14	Epidemic	2/19/13	BAL	ST512
KpVA-3	Sporadic	3/14/13	B	ST258
KpVA-15	Sporadic	3/24/13	U	ST512
KpVA-16	Sporadic	3/24/13	U	ST512

[a] B, blood; WS, wound sample; SP, sputum; BAL, bronchoalveolar lavage fluid; TA, tracheal aspirate; U, urine.

***In silico* MLST and gene mining.** Multilocus sequence typing (MLST) profiles were obtained *in silico* by analyzing appropriate gene variants (http://bigsdb.web.pasteur.fr/perl/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_seqdef_public&page=downloadAlleles) for each genome, using an in-house Python script. The presence of selected genes coding for antibiotic resistance and virulence factors was determined by using BLAST with a specifically designed database, BIGSdb-Kp (http://bigsdb.web.pasteur.fr/perl/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_seqdef_public&page=sequenceQuery) (19). All hits were manually checked, and genes requiring specificity for a particular variant (e.g., bla_{KPC} versus bla_{OXA-48}) were requested to have 100% identity with the database sequences. BLAST searches and filters were also used to test for the presence of yersiniabactin genes in all genomes used for the global phylogenetic analysis (see Results for details). Analysis of the presence of insertion sequences within the *mgrB* gene (a putative determinant of colistin resistance) (32) was performed with a manually corrected BLAST search.

Core SNP detection and phylogeny. Whole-genome sequences of the 16 isolates were added to a previously described database of 319 genomes of *K. pneumoniae* strains isolated throughout the world (21). Single-nucleotide polymorphisms (SNPs) were detected using an in-house pipeline based on Mauve software (33), using the published NJST258_1 complete genome as a reference (21). Briefly, each genome was individually aligned with the reference and alignments were merged with Perl scripts to obtain a global alignment. Core SNPs, defined as single-nucleotide variations flanked by at least one identical nucleotide on both sides in all genomes analyzed (34), were detected. Maximum likelihood phylogenetic analysis was performed using core SNPs merged in a multialignment file. RAxML software was used (35) with the generalized time-reversible (GTR) model and 100 bootstraps.

Core genome MLST. Core genome MLST (cgMLST) analysis was performed using the BIGSdb software and database (19, 36). cgMLST profiles made of allelic variants at 694 loci were obtained for 219 genomes of CG258, representing the 16 genomes presented in this work. cgMLST profiles were used to produce a tree of all 219 genomes, using the unweighted pair group method with arithmetic mean (UPGMA) approach.

Outbreak reconstruction. The spreading routes of outbreak strains were reconstructed by combining core SNPs and the dates of sample collection, applying the SeqTrack method implemented in the R package Adegenet (37). The outbreak chain of transmission was then obtained using the R package Outbreaker (38).

Nucleotide sequence accession number. Genome assemblies were deposited in the EMBL database under accession number PRJEB7661.

RESULTS

Species identification and antimicrobial susceptibility. In this work, the first isolate obtained from each patient was investigated. Species identification was performed with biochemical and molecular assays. The seven isolates collected in February 2013 were suspected to belong to a single outbreak; these strains are referred to as epidemic. The remaining isolates are termed sporadic. Five of 16 isolates were obtained from blood cultures (3 of 7 for patients involved in the ICU outbreak).

Phenotypic assays detected resistance to imipenem, meropenem, and ertapenem in all isolates, regardless of the isolation date. All were thus classified as carbapenem resistant. The seven epidemic isolates were resistant to colistin but susceptible to aminoglycosides (gentamicin, amikacin, and tobramycin). Three of 9 sporadic isolates were also resistant to colistin.

Drug resistance determinants and virulence factors. Isolates were subjected to a set of PCR assays to detect drug resistance genes and virulence factors. The bla_{KPC} gene was detected in all isolates, while other carbapenem resistance genes (bla_{NDM}, bla_{IMP-1}, bla_{OXA-48}, and bla_{VIM}) were not detected. Genes coding

TABLE 2 Antimicrobial susceptibility profiles of 16 investigated *K. pneumoniae* isolates

Characteristic	KPVA-4	KPVA-5	KPVA-6	KPVA-7	KPVA-1	KPVA-2	KPVA-8	KPVA-10	KPVA-9	KPVA-11	KPVA-12	KPVA-13	KPVA-14	KPVA-3	KPVA-15	KPVA-16
Date of isolation (mo/day/yr)	1/11/11	1/28/11	3/14/11	5/3/11	10/31/12	1/30/13	2/1/13	2/8/13	2/12/13	2/13/13	2/15/13	2/16/13	2/19/13	3/14/13	3/24/13	3/24/13
Sensitivity and MIC (mg/liter)[a]																
Ampicillin	R, >16	R, >16	R, >16	R, >16	R, >8	R, >8	R, >8	R, >8	R, >8	R, >8	R, >8	R, >8	R, >8	R, >8	R, >8	R, >8
Amoxicillin-clavulanate	R, >16/8	R, >16/8	R, >16/8	R, >16/8	R, >8/2	R, >8/2	R, >8/2	R, >8/2	R, >8/2	R, >8/2	R, >8/2	R, >8/2	R, >8/2	R, >8/2	R, >8/2	R, >8/2
Ceftazidime	R, >16	R, >16	R, >16	R, >16	R, >8	R, >8	R, >8	R, >8	R, >8	R, >8	R, >8	R, >8	R, >8	R, >8	R, >8	R, >8
Cefotaxime	R, >32	R, >32	R, >32	R, >32	R, >4	R, >4	R, >4	R, >4	R, >4	R, >4	R, >4	R, >4	R, >4	R, >4	R, >4	R, >4
Aztreonam	R, >16	R, >16	R, >16	R, >16	R, >16	R, >16	R, >16	R, >16	R, >16	R, >16	R, >16	R, >16	R, >16	R, >16	R, >16	R, >16
Ertapenem	R, >1	R, >32	R, >1	R, >1	R, >1	R, >1	R, >32	R, >32	R, >32	R, >32	R, >32	R, >32	R, >32	R, >1	R, >1	R, >1
Imipenem	R, >32	R, 8	R, 32	R, 8	R, >8	R, >8	R, >32	R, >32	R, >32	R, >32	R, >32	R, >32	R, >32	R, 8	R, >8	R, >8
Meropenem	R, >32	R, 32	R, >32	I, 6	R, >8	R, >8	R, >32	R, >32	R, >32	R, >32	R, >32	R, >32	R, >32	R, 12	R, >8	R, >8
Ciprofloxacin	R, >2	R, >2	R, >2	R, >2	R, >1	R, >1	R, >1	R, >1	R, >1	R, >1	R, >1	R, >1	R, >1	R, >1	R, >1	R, >1
Levofloxacin	R, >4	R, >4	R, >4	R, >4	R, >2	R, >2	R, >2	R, >2	R, >2	R, >2	R, >2	R, >2	R, >2	R, >2	R, >2	R, >2
Amikacin	R, 48	R, 64	R, 48	R, 64	R, >16	S, 1	S, 1	S, 1	S, 1	S, 1	S, 1	S, 1	S, 1	R, 48	R, >16	R, >16
Gentamicin	S, 0.5	S, 1.5	S, 1	S, 2	S, 2	S, 0.25	S, 0.25	S, 0.25	S, 0.25	S, 0.25	S, 0.25	S, 0.25	S, 0.25	S, 1.5	S, 4	S, 2
Tobramycin	S, 0.5	R, 16	R, 16	R, 24	R, >4	R, >4	S, 0.38	S, 0.38	S, 0.38	S, 0.38	S, 0.38	S, 0.38	S, 0.38	R, 12	R, >4	R, >4
Colistin	S, 0.12	S, 0.19	S, 0.38	S, 0.19	R, >4	S <1	R, 8	R, 8	R, 8	R, 8	R, 8	R, 8	R, 8	S, 0.19	R, >4	R, >4

[a] R, resistant; I, intermediate; S, susceptible.

for type 1 and type 3 fimbriae (*fim* and *mrk* operons, respectively) were detected in all isolates, as was the enterobactin siderophore located in the *ent* operon (Table 3). Three sporadic isolates carried genes for yersiniabactin, i.e., *ybtS* and the iron-repressible genes *irp1* and *irp2* (14, 39, 40). In *K. pneumoniae*, the yersiniabactin siderophore is expressed together with or instead of enterobactin. Finally, genomes were scanned for the presence of any beta-lactamase gene with the web tool BIGSdb (19). Genes coding for Bla_{SHV} were detected in all isolates, while genes coding for Bla_{TEM} were detected in 13 of the 16 genomes, being absent only in KpVa-2, KpVA-3, and KpVA-4. None of the analyzed genomes were found to carry genes of any of the other 17 beta-lactamase families.

Whole-genome sequencing and characterization. Whole-genome sequences were obtained for all 16 *K. pneumoniae* isolates. The assembled genomes were characterized *in silico* for MLST and were searched for genes coding for drug resistance determinants and virulence factors (Tables 1 and 3). MLST analysis enabled identification of two groups of isolates. Six isolates (KpVA-2, KpVA-3, KpVA-4, KpVA-5, KpVA-6, and KpVA-7) were of sequence type 258 (ST258); 10 isolates, including the epidemic ones, belonged to ST512. ST512 differs from ST258 at a single nucleotide, thus belonging to the same clonal group, CG258 (19, 20). Confirming the results obtained by molecular analysis, all strains were found to carry the bla_{KPC} gene. The bla_{KPC2} variant (KPC2) was found in three of six isolates belonging to ST258, while the remaining 13 isolates carried the bla_{KPC3} variant (KPC3).

The three isolates belonging to ST258 and possessing the bla_{KPC2} variant also presented unique profiles of virulence and drug resistance factors. Strains KpVA-2, KpVA-3, and KpVA-4 harbored the *irp1*, *irp2*, *ybtA*, and *ybtS* genes, which were not detected in other strains. These four genes encode yersiniabactin, a virulence factor expressed by *Yersinia* and other enterobacteria, including *K. pneumoniae* (19, 41). All 16 strains analyzed here possessed the *mrk* and *fim* operons, coding for fimbrial genes (11, 12), and the *ent* operon, coding for enterobactin (14), consistent with previous results showing that these genes are highly conserved in *K. pneumoniae* (19, 30). None of the isolates demonstrated *rmpA* and *wzy*-K1 (*magA*) genes, which are hypermucoviscosity-associated genes (16, 17, 19).

Genes related to colistin resistance were also investigated. The entire set of *pmr* genes was highly conserved among the 16 strains, including the *pmrB* locus, which has been indicated as a colistin resistance determinant (42). All colistin-resistant strains harbored a variant of the *mgrB* gene interrupted by an IS*5*-like transposon (Table 3). Insertion of a transposon in this gene has been reported as a determinant of colistin resistance (32).

Global core SNP phylogeny. A global genome phylogeny of *K. pneumoniae*, including the 16 isolates investigated in this study, was obtained by adding the novel genomes to a previously constructed database of 319 isolates (21). Phylogeny was obtained in order to contextualize our strains among the previously sequenced *K. pneumoniae* isolates. The 16 novel genomes clustered in 5 monophyletic groups on the global tree (Fig. 2). As expected, they fit within CG258. Interestingly, these 16 isolates were assigned to three of the four previously identified groups of Italian isolates of CG258 (21). Inclusion of the investigated genomes in the global phylogeny allowed us to define the relationships among the isolates at the investigated hospital. KpVA-2, KpVA-3, and KpVA-4, the three isolates that were yersiniabactin positive, clus-

TABLE 3 Antimicrobial resistance and virulence determinants in 16 investigated *K. pneumoniae* isolates

Characteristic	KPVA-4	KPVA-5	KPVA-6	KPVA-7	KPVA-1	KPVA-2	KPVA-8	KPVA-10	KPVA-9	KPVA-11	KPVA-12	KPVA-13	KPVA-14	KPVA-3	KPVA-15	KPVA-16
Date of isolation (mo/day/yr)	1/11/11	1/28/11	3/14/11	5/3/11	10/31/12	1/30/13	2/1/13	2/8/13	2/12/13	2/13/13	2/15/13	2/16/13	2/19/13	3/14/13	3/24/13	3/24/13
Presence and type of antibiotic resistance determinants																
bla_{KPC}	2	3	3	3	3	2	3	3	3	3	3	3	3	2	3	3
bla_{VIM}	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
bla_{NDM1}	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
bla_{IMP}	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
bla_{OXA}	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
bla_{SHV}	12	11	11	11	11	12	11	11	11	11	11	11	11	11	11	11
bla_{TEM}	No	1	1	1	1	No	1	1	1	1	1	1	1	No	1	1
mgrB insertion	No	No	No	No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes
Presence of virulence determinants																
fimACDEFH	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
mrkABCDF	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
rpmA	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
magA	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
entABCDEF	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
ybtA	Yes	No	No	No	No	Yes	No	No	No	No	No	No	No	Yes	No	No
ybtS	Yes	No	No	No	No	Yes	No	No	No	No	No	No	No	Yes	No	No
irp1	Yes	No	No	No	No	Yes	No	No	No	No	No	No	No	Yes	No	No
irp2	Yes	No	No	No	No	Yes	No	No	No	No	No	No	No	Yes	No	No

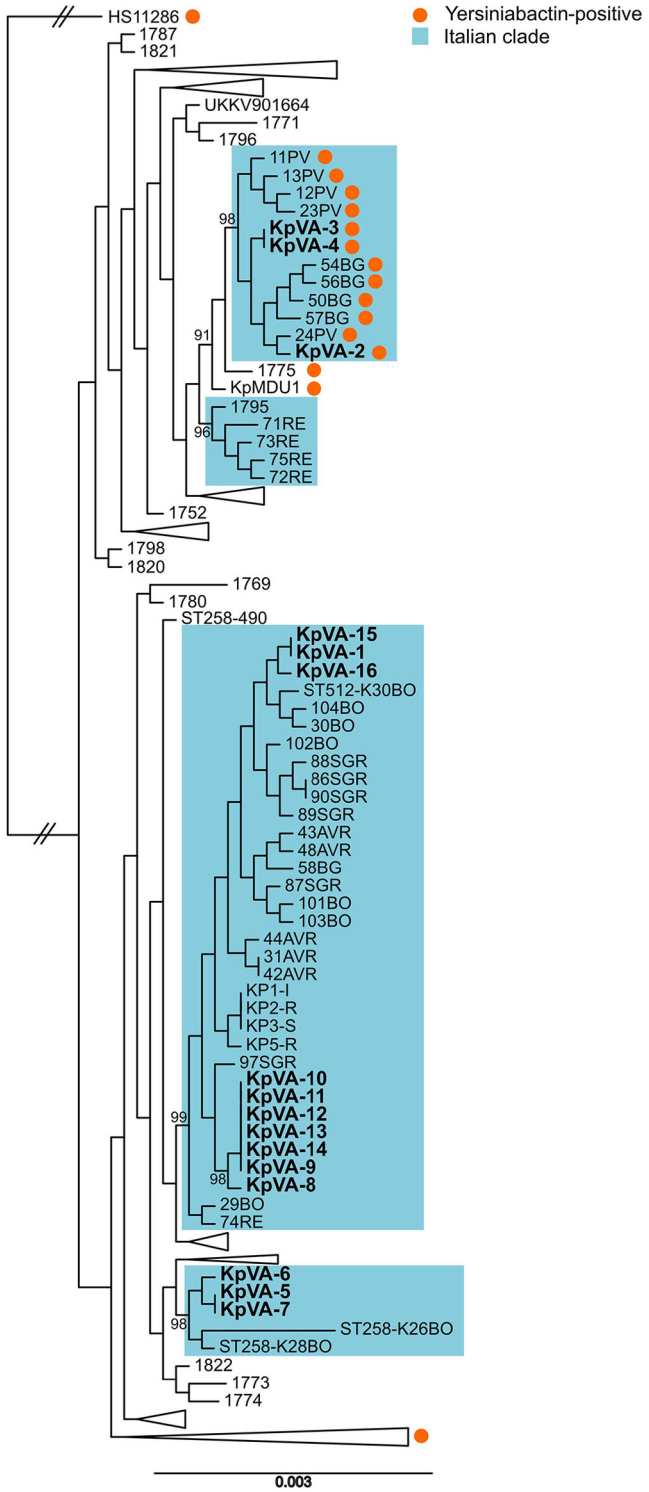

FIG 2 Representation of the phylogenetic relationships between isolates of clonal group 258 of *Klebsiella pneumoniae*, reconstructed using RaxML software with 100 bootstrap replicates and the generalized time-reversible model. The 16 novel isolates investigated in this study are highlighted in bold type. Highlighted in blue boxes are the four clades encompassing Italian isolates (both newly sequenced and taken from databases). Triangles represent coherent monophyletic clades of isolates from other countries, and orange dots indicate the presence of yersiniabactin genes. Bootstrap values are indicated only on nodes of interest, for the sake of image clarity. For a complete phylogeny of 335 *K. pneumoniae* isolates worldwide, see Fig. S1 in the supplemental material.

tered together in a clade containing nine additional Italian strains and two U.S. strains, all demonstrating the yersiniabactin genes (which could also contribute to copper toxicity) (38). Thirty-nine additional isolates, belonging to different sequence types and scattered on the global *K. pneumoniae* phylogeny, also demonstrated yersiniabactin. The genomes of the seven isolates collected in February 2013 and hypothesized to represent a single epidemic event clustered together in a single, well-supported, phylogenetic clade (Fig. 2). This result confirmed the original hypothesis of a single clone being responsible for the seven infections that occurred in the ICU.

In analyses of the numbers of SNPs differentiating the isolates, the seven strains belonging to the investigated outbreak presented an average of 20 SNPs per genome in comparisons among them. Interestingly, a similar average number (27 SNPs per genome) differentiated strains KpVA-1, KpVA-15, and KpVA-16, which are grouped in a single clade but have been sampled over a longer time (about 5 months). This could indicate a difference in the measured paces of the molecular clock between the two clusters. Multiple hypotheses could explain the observed situation, such as the presence of different environmental conditions or a conservative pressure from purifying selection.

Core genome MLST. cgMLST analysis was performed with the same genomic data set used for the SNP phylogeny presented in Fig. 2 (219 *K. pneumoniae* genomes belonging to CG258). The resulting UPGMA tree (see Fig. S2 in the supplemental material) is largely consistent with the tree resulting from the SNP-based phylogenomic analysis. Specifically, in both analyses, the main subdivisions of CG258 (23) were clearly detectable, while the 16 genomes presented in this work were clustered in four monophyletic groups, one of which corresponded to the seven outbreak strains.

Outbreak reconstruction. To define the investigated ICU outbreak in greater detail, a genomic network was built using the core SNPs identified among the epidemic isolates, which were ordered according to the isolation date. The resulting structure showed a star-like topology (Fig. 3) centered around the isolate obtained from patient zero (KpVA-8). This structure suggested a nonlinear spread of infection. Thus, multiple events of contagion probably took place, all starting from patient zero and infecting six ICU patients (Fig. 3). It is important to note that patient zero (KpVA-8) stayed in the ICU for >2 months and the stays of the other infected patients coincided with his presence (Fig. 1). In addition, the location of his bed in the ward was not related to the date of infection (i.e., patients in beds closer to the bed of patient zero were not infected before patients in beds more distant from the bed of patient zero). Interestingly, the phylogenetic analysis of SNPs (Fig. 2) showed KpVA-8 (i.e., the isolate from patient zero) as the sister taxon of a single clade including the other six epidemic strains (Fig. 2). The result confirms that isolate KpVA-8 was at the origin of the outbreak.

DISCUSSION

This work was aimed at characterizing 16 carbapenem-resistant *K. pneumoniae* isolates collected from a single hospital during a 27-month period, including an epidemic that occurred in February 2013. This allowed us to identify the genomic characteristics of the isolates, to elucidate the epidemiological relationships among them, and to place them in the context of the global phylogeny of *K. pneumoniae*. In particular, whole-genome analyses allowed us to characterize the diversity of this bacterial species within a single

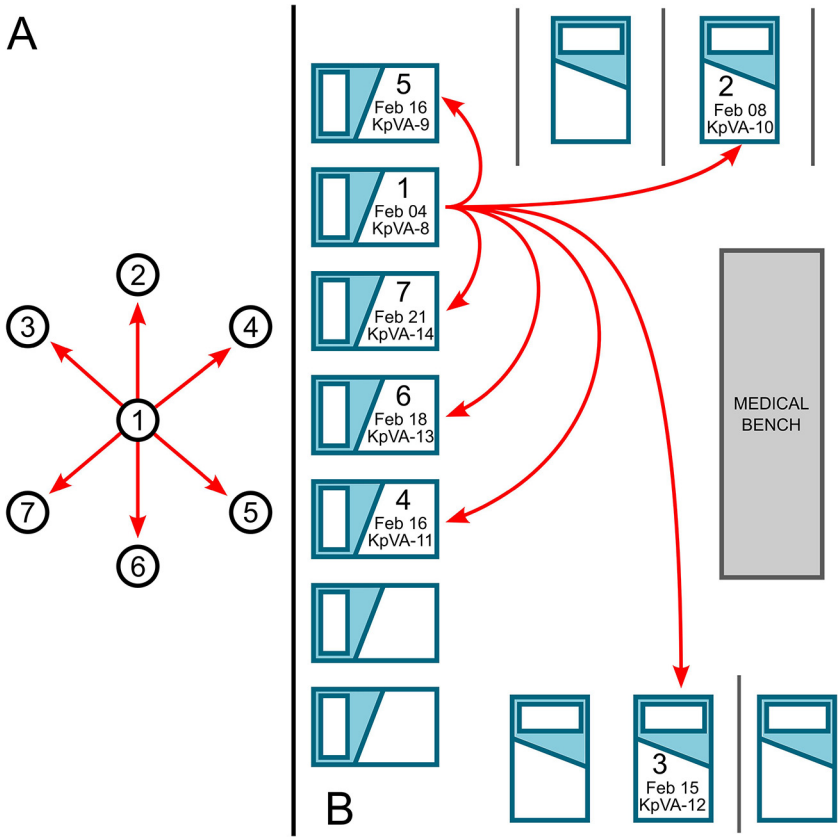

FIG 3 (A) Reconstruction of the star-like diffusion pattern, starting from isolate KpVA-8, among the seven *K. pneumoniae* isolates belonging to a single outbreak event that occurred in the ICU of Ospedale di Circolo e Fondazione Macchi in February 2013. The star-like topology was obtained using the R package Outbreaker. Numbers in bold indicate the temporal order of contagion. (B) Graphic representation of the bed-to-bed spread of infection on a map of the ICU.

hospital, to contextualize the local features within the global genomic spectrum of the species, and to reconstruct the spreading route of seven isolates within the ICU.

The 16 carbapenem-resistant isolates were shown to belong to CG258, the most prevalent KPC-producing *K. pneumoniae* lineage. Indeed, all 16 genomes demonstrated the gene bla_{KPC}, and none of them had other known carbapenem resistance genes. This is not surprising, considering previous reports that showed the worldwide diffusion and high prevalence of KPC isolates of this clonal group among carbapenem-resistant *K. pneumoniae* strains (21, 23).

When the drug resistance profiles of the investigated isolates were compared, the main difference was colistin resistance. In 10 of the 16 isolates, the colistin MIC was 4 mg/liter or higher (Table 2), thus above the EUCAST MIC breakpoint. Genomic analysis aimed at detecting the determinants for this resistance trait found that the 10 resistant isolates demonstrated insertion of an IS*5*-like transposon in the *mgrB*, a gene that regulates a pathway of lipopolysaccharide biosynthesis (43). Insertion of an IS*5*-like sequence in the *mgrB* gene has indeed been proposed as a determinant of colistin resistance (32). Our results appear to support this causative link, considering that none of the six colistin-sensitive strains presented the aforementioned insertion.

The global phylogeny of *K. pneumoniae* (Fig. 2) reveals that the 16 isolates are assigned to three of the four previously characterized Italian clades of CG258. These four clades have been proposed to represent four different dissemination events for KPC isolates in Italy, from 2008 to 2010 (21). Therefore, within a time span of 27 months, three of the four main Italian lineages of CG258 KPC-producing *K. pneumoniae* were detected in a single hospital in northwestern Italy. This result clearly indicates that at least three of the four lineages are currently circulating, creating a scenario of multiple, contemporary, overlapping epidemics.

With regard to virulence genes, all 16 isolates possessed the operons *fim*, *ent*, and *mrk* but lacked the genes *rmpA* and *wzy*-K1 (*magA*). The only detected difference among the 16 isolates was the presence in three isolates of four genes responsible for yersiniabactin synthesis (*ybtA*, *ybtS*, *irp1*, and *irp2*). Yersiniabactin has been reported to provide advantages in metabolism and multiplication, particularly in mixed infections and under iron-deprived conditions and especially in pulmonary infections, according to recent studies (14, 39, 40). When investigating the presence of yersiniabactin genes in the global CG258 *K. pneumoniae* phylogeny (Fig. 1), we detected a monophyletic group encompassing the three novel isolates as well as all other Italian isolates belonging to the same subclade, in addition to two U.S. isolates. This result clearly indicates that these genes were acquired before the diversification of this specific subclade and have been maintained since, which allows the characterization of this group of isolates as a yersiniabactin-positive monophyletic lineage of strains within CG258.

Seven of the 16 isolates, which were collected from ICU pa-

tients over a period of 17 days, were hypothesized to represent a single epidemic event. The monophyletic relationships among the seven epidemic isolates (Fig. 2) confirmed the hypothesis. A specific analysis for outbreak reconstruction showed that the epidemic isolates were connected in a star-like diagram (Fig. 3) originating from the isolate from patient zero. This is congruent with the rapid spread among the seven ICU patients.

Epidemic isolates could not be differentiated from sporadic isolates based on a specific pattern of the presence/absence of genes coding for virulence factors. The drug resistance profiles of these isolates also were identical to those of some sporadic isolates. This indicates that the spread of the outbreak was not related to genes conferring a specific advantage to the epidemic clone. Rather, it suggests that external factors might have caused the spread of the clone among ICU patients.

In conclusion, this study shows how whole-genome analysis can facilitate accurate reconstruction of the spread of bacterial pathogens. The wealth of data from genome sequencing allows reconstruction of the relationships of isolates from single hospitals and outbreaks and placement of the isolates in overall global phylogenies, and comparisons of genomes permit determination of whether strains involved in a specific outbreak share common characteristics that might confer specific selective advantages. The introduction of bacterial genomics into clinical settings will thus allow reconstruction of the routes and causes of nosocomial infections, with estimation of the relative roles of human- and microbe-related factors.

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We declare that we have no conflicts of interest to report.

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