Resistome and virulome accretion in an NDM-1-producing ST147 sublineage of *Klebsiella pneumoniae* associated with an outbreak in Tuscany, Italy: a genotypic and phenotypic characterisation

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Summary

Background Carbapenemase-producing Enterobacterales (CPE), particularly those producing metallo-β-lactamases, are among the most challenging antibiotic-resistant pathogens, causing outbreaks of difficult-to-treat nosocomial infections worldwide. Since November 2018, an outbreak of New Delhi metallo-β-lactamases-positive CPE (NDM-CPE) has emerged in Tuscany, Italy. In this study, we aimed to investigate the NDM-CPE associated with the outbreak and characterise the responsible *Klebsiella pneumoniae* clone.

Methods We used whole-genome sequencing and bioinformatic analysis to characterise NDM-CPE isolates that caused bloodstream infections in 53 patients at 11 hospitals in Tuscany and that were collected between Jan 1, 2018, and July 5, 2019 (ie, the early phase of the outbreak and preceding months). The CPE isolates characterised in this study were isolated and identified at the species level and as NDM producers by six diagnostic microbiology laboratories that serve the 11 hospitals. We used comparative genomic analysis, antimicrobial susceptibility testing, plasmid conjugal transfer assays, evaluation of virulence potential in the *Galleria mellonella* infection model, and serum bactericidal assays to further characterise the clone causing the outbreak.

Findings The outbreak was sustained by an ST147 *K pneumoniae* producing NDM-1, which had a complex resistome that mediated resistance to most antimicrobials (except cefiderocol, the aztreonam–avibactam combination, colistin, and fosfomycin). The clone belonged to a sublineage of probably recent evolution, occurred by the sequential acquisition of an integrative and conjugative element encoding the yersiniabactin siderophore, an FIB(pQil)-type multiresistance plasmid carrying bla_{NDM-1}, and a transferable chimeric plasmid, derived from virulence elements of hypervirulent *K pneumoniae*, carrying several resistance and virulence determinants. Infection of *G mellonella* larvae revealed a variable virulence potential. The behaviour in serum bactericidal assays was different from typical hypervirulent *K pneumoniae* strains, with variable grades of serum resistance apparently associated with mutations in specific chromosomal loci (*csrD*, *pal*, and *ramR*).

Interpretation This description of a sublineage of ST147 *K pneumoniae* with a complex resistome and virulome that is capable of sustaining a large regional outbreak adds to existing research on the evolutionary trajectories within high-risk clones of *K pneumoniae*. Global surveillance programmes are warranted to track the dissemination of these lineages, and to prevent and control their spread.

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Introduction

Antimicrobial resistance is a major public health concern escalating worldwide. Carbapenemase-producing Enterobacterales (CPE) are considered among the most challenging antibiotic-resistant pathogens because they have multidrug-resistant profiles that leave very few treatment options. As such, CPE are a typical cause of difficult-to-treat infections associated with high morbidity and mortality rates.[1]

The introduction of new β-lactamase inhibitor combinations, such as ceftazidime–avibactam, imipenem–relebactam, and meropenem–vaborbactam, provided a major breakthrough for treatment of CPE infections. However, these new β-lactamase inhibitor combinations do not cover strains producing metallo-β-lactamases.[2] For this reason, the dissemination of CPE-producing metallo-β-lactamases remains a matter of great concern. On a global scale, New Delhi metallo-β-lactamase

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Research in context

Evidence before this study

Since November 2018, a large outbreak of Enterobacterales producing New Delhi metallo-β-lactamase (NDM)-type carbapenemases emerged in Tuscany, Italy, largely sustained by a NDM-1-producing *Klebsiella pneumoniae* clone of ST147. On July 1, 2021, we searched PubMed with the following queries: "(((*klebsiella pneumoniae*) AND (outbreak OR epidemic)) AND (NDM or blaNDM)) AND (ST147 OR sequence type 147)", "(((*klebsiella pneumoniae*) AND (virulent OR virulence)) AND (outbreak OR epidemic)) AND (ST147)", "((*klebsiella pneumoniae*) AND (virulence OR hypervirulent)) AND (ST147)", "((virulence OR hypervirulent) AND (*klebsiella pneumoniae*) AND (NDM or blaNDM))", without date or language restrictions. A total of 199 non-duplicate articles (excluding reviews and perspective papers [n=13]) were identified by this search strategy. Among these, we found four studies reporting on prolonged large outbreaks (in 2015–19) in Poland and the USA, or smaller clusters in Spain and Tunisia (in 2010–15), caused by ST147 *K pneumoniae* producing NDM-1 and carrying the yersiniabactin siderophore. Only three publications described the sporadic emergence of NDM-1-producing ST147 *K pneumoniae* carrying acquired virulence determinants typical of hypervirulent *K pneumoniae*, including aerobactin and regulators of the mucoid phenotype, in the UK (2018–19) and Egypt (2018). Another study reported on the characterisation of *K pneumoniae* isolates of different sequence types (ST15, ST147, ST395, and ST874) carrying hybrid plasmids coharbouring virulence genes and bla_{NDM}, collected in Russia (2017), which were highly related to the hybrid element detected in hypermucoviscous ST307 *K pneumoniae* isolates, carrying bla_{NDM-1} and bla_{OXA-48}, associated with a 2019 outbreak in Germany. None of the aforementioned studies reported on outbreaks sustained by NDM-1-producing ST147 *K pneumoniae* carrying acquired virulence determinants typical of hypervirulent *K pneumoniae*, evaluated the transfer abilities of emerging hybrid plasmids combining resistance and virulence determinants, or evaluated the possible modulation of the virulence potential of this *K pneumoniae* lineage in an outbreak context.

Added value of this study

In this study, we highlighted the remarkable epidemic potential of a novel ST147 sublineage of *K pneumoniae* of likely recent origin, characterised by a complex resistome accounting for resistance to most antimicrobials and by a peculiar virulome typical of hypervirulent *K pneumoniae*, that so-far had only been described in association with sporadic cases. We delineated the likely evolutionary dynamics of this sublineage within ST147, expanding current knowledge about the mechanisms of virulence and resistance evolution within this high-risk clone of *K pneumoniae*. Additionally, we described the accessory genome involved in the evolution of this sublineage and demonstrated the transferability potential of a chimeric plasmid carrying several resistance and virulence determinants, derived from virulence elements of hypervirulent *K pneumoniae*. Finally, we evaluated the virulence potential of this sublineage in a *Galleria mellonella* infection model and by serum bactericidal assays, showing a heterogeneous intra-clonal behaviour that was possibly explained by mutations in specific chromosomal genes as a result of different microevolutionary trajectories following clonal expansion.

Implications of all the available evidence

Convergence of extensive resistance (as that mediated by metallo-β-lactamases and other genes) and virulence in epidemic clones of *K pneumoniae* represents an alarming evolutionary development. In a context where the high plasticity of these lineages might allow for a modulation of virulence through mutational events, the acquisition of hybrid transferable plasmids carrying both resistance and virulence determinants, which can simultaneously mediate the dissemination of both traits, represents an additional concern. The available evidence underscores the potential of similar clones to cause even large epidemics and the need to trace their dissemination, since early detection and continuous surveillance represent key actions to prevent the spread of these pathogens in the health-care system.

(NDM) enzymes are overall the most prevalent type of metallo-β-lactamases among CPE, with a notable ability for horizontal dissemination and propensity to be associated with successful clonal lineages of *Enterobacterales*.[3]

During the past decade, CPE have become endemic in several countries, with Italy being one of the most affected in Europe.[4] Since 2010, a rapid increase of CPE infections was observed in Italy, leading to high endemicity of carbapenemase-producing *Klebsiella pneumoniae*. This endemicity was mostly caused by the successful clonal expansion of a *K pneumoniae* carbapenemase (KPC)-producing *K pneumoniae* clone and belonging to clonal complex 258.[5] Within the past decade, clonal complex 258 has been compounded by the emergence of additional KPC-producing *K pneumoniae* clones (eg, ST307 and ST101),[6] whereas CPE producing other types of carbapenemases (eg, OXA-48 and metallo-β-lactamases) have remained sporadic or at most been responsible for small outbreaks.[7]

Since November 2018, however, a large outbreak of CPE-producing NDM-type carbapenemases (NDM-CPE) emerged in Tuscany, Italy.[7,8] Preliminary characterisation of subsets of NDM-CPE causing invasive infections collected during emergence of the outbreak, in the frame of studies focused on clinical, epidemiological, and infection control aspects, revealed that the outbreak was mostly associated with the clonal expansion of an NDM-1-producing sequence type (ST) 147 lineage of *K pneumoniae*, with sporadic detection of isolates of other species (*Escherichia coli*) and other lineages of *K pneumoniae* producing NDM-type enzymes.[7,9]

In this study we report the characterisation of the NDM-CPE causing invasive infections collected during emergence of the outbreak, with a focus on the genotypic and phenotypic characteristics of the NDM-1-producing ST147 *K pneumoniae* sustaining the outbreak.

Methods

Study design and setting

This study was a retrospective microbiological characterisation of 54 NDM-CPE collected from episodes of bloodstream infections observed among hospitalised patients in Tuscany, Italy, between Jan 1, 2018, and July 5, 2019 (ie, the early phases of the outbreak reported in Tuscany since November 2018,[7] and the preceding months). According to the Regional Health Agency of Tuscany, a total of 60 bloodstream infections caused by NDM-CPE were reported in Tuscany during the study period. Isolates of NDM-CPE from 54 (90%) of 60 bloodstream infections were available for further characterisation and included in this study; 50 (93%) of 54 isolates were *K pneumoniae* and 4 (7%) were *E coli*. Overall, these 54 isolates were collected from 53 patients (37 [70%] men; 16 [30%] women), with one *K pneumoniae* isolate and one *E coli* isolate being from two subsequent bloodstream infections that occurred in the same patient. Patients were admitted to 11 regional hospitals in Tuscany during the study period (figure 1A).

The 54 NDM-CPE characterised in this study were isolated and identified at the species level and as NDM producers by the six diagnostic microbiology laboratories (Florence Careggi, Lucca, Livorno, Pisa, Pontedera, and Siena) serving the 11 regional hospitals where the patients were admitted (appendix 1 p 2), and were sent to the laboratory in Florence for further characterisation by whole-genome sequencing, to confirm identification and investigate genotypic diversity. 48 (89%) isolates of NDM-1-producing ST147 K pneumoniae were found to be clonally related and identified as primarily involved in the outbreak. These isolates were then subjected to further characterisation including: (1) antimicrobial susceptibility and resistome profiling, (2) analysis of the accessory genome, (3) virulome profiling and, for a subset of the isolates, including representatives of the different plasmidome profiles (appendix 1 p 3), characterisation of the virulence behaviour in a Galleria mellonella infection model and of resistance to human serum, and (4) comparative genomic analysis with a global collection of ST147 K pneumoniae to gather insights about the evolutionary history of this clone.

Ethical approval was not required for this study since it was a retrospective study of bacterial isolates.

Laboratory methodology

Confirmed NDM-CPE were sent to a reference laboratory (Florence Careggi University Hospital, Florence, Italy), where isolates were stored at –80°C in Brain Heart Infusion broth (Oxoid, Hampshire, UK) containing 20% v/v glycerol (Sigma Aldrich, Saint Louis, MO, USA) for further characterisation. The glycerol stocks were used as sources of the isolates for all the experiments performed in this study (appendix 1 p 2).

Antimicrobial susceptibility testing was performed by reference broth microdilution or by agar dilution methods according to the International Standards Organization standard (reference ISO 20776-1:2019; appendix 1 p 2).[10]

Results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing clinical breakpoints (version 11.0).

For EUCAST clinical breakpoints see https://www.eucast.org/clinical_breakpoints/

The string test for hypermucoviscous phenotype was performed after culturing bacteria on MacConkey agar plates (Oxoid) at 37°C for 18 h, and using an inoculation loop to generate a viscous string more than 5 mm in length from a single bacterial colony (positive test), as previously described.[11]

All the 54 NDM-CPE investigated in this study were sequenced with Illumina technology (Illumina, San Diego, CA, USA) and a subset were also investigated using the Oxford Nanopore long-read sequencing approach (appendix 1 p 3).

Plasmid transfer experiments were performed with six NDM-1-producing ST147 *K pneumoniae* representative of different plasmidome profiles, as previously described (appendix 1 p 3).[12]

The virulence potential of selected NDM-1-producing ST147 *K pneumoniae* was studied using a *Galleria mellonella* infection model and by serum bactericidal assays (appendix 1 p 4).

Data sources

Epidemiological data (ie, incidence of bloodstream infections caused by CRE and NDM-CPE, 30-day mortality rates associated with bloodstream infections caused by NDM-CPE, and patients' sex) were from the Regional Health Agency of Tuscany, which developed a data collection form to track microbiologically confirmed cases of NDM-CPE in November, 2018, within the national surveillance programme on CPE mandated by the Italian Ministry of Health (circulars number 35470 [Dec 6, 2019] and 4968 [Feb 26, 2013]), that was used to retrospectively and prospectively collect the data.

See **Online** for appendix 1

Statistical analysis

Statistical analyses were executed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). The 30-day mortality rates for bloodstream infections caused by NDM-CPE were defined as the ratio of all-cause deaths to the total number of cases occurring in-hospital within 30 days of admission; 95% CIs were calculated as measure of uncertainty. Single nucleotide polymorphism (SNP) values are presented as range, mean (SD), and median (IQR). For *G mellonella* experiments, survival curves were evaluated with the Kaplan-Meyer estimator, and differences in the survival trends were evaluated using the log-rank Mantel–Cox test. The lethal dose

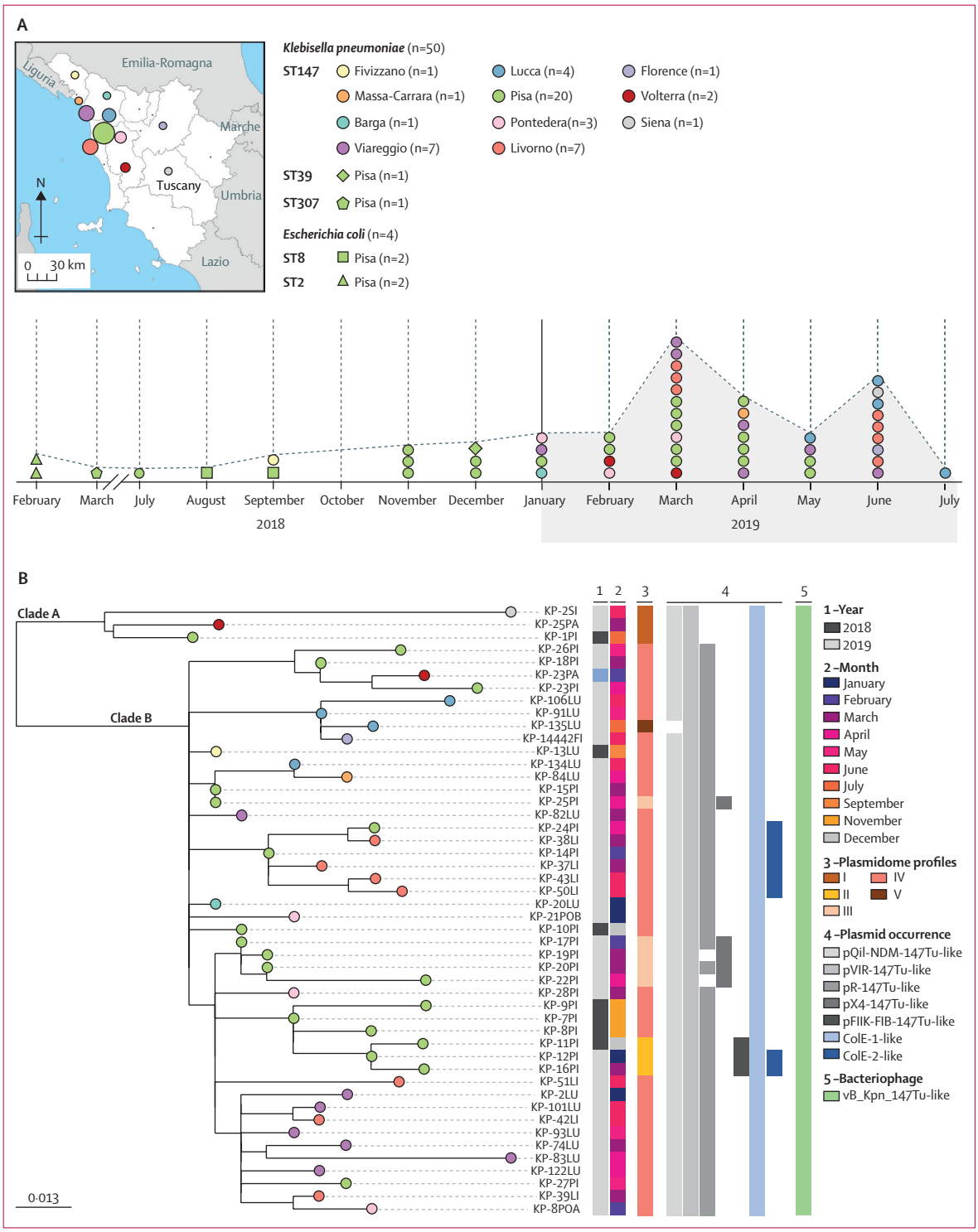

Figure 1: **Features of the NDM-CPE emerging in Tuscany, Italy**
Data is from Jan 1, 2018, to July 5, 2019 (ie, the early phases of the outbreak and the preceding months). (A) Geographical distribution of the health-care facilities involved in the outbreak and isolation timeframe of the NDM-CPE (n=54), including NDM-1-producing ST147 (n=48), ST39 (n=1), and NDM-5-producing ST307 (n=1) *Klebsiella pneumoniae*, and ST2 (n=2) and ST8 (n=2) *Escherichia coli*. Source health-care facilities are represented by different colours, and bacterial clones (ie, ST) are shown in different shapes. The size of each circle on the map is proportional to the number of bacterial isolates provided by each hospital. (B) Phylogenetic relatedness of NDM-1-producing ST147 *K pneumoniae* (n=48) characterised in this study. Details about the isolation (ie, year and month), the plasmidome profile, and the occurrence of the different plasmids identified in the outbreak clone has been provided. The colours of the isolate tips correspond to the associated health-care facility. CPE=carbapenemase-producing Enterobacterales. NDM=New Delhi metallo-β-lactamases. ST=sequence type.

concentrations inducing 50% larval death (lethal dose 50% [LD_{50}) were calculated using a non-linear regression analysis in GraphPad Prism 7, and differences in LD_{50} were evaluated using the extra sum-of-squares *F* test. For serum bactericidal assays, the statistically significant differences in viable counts were assessed using the unpaired *t* test (two-tailed). Statistical significance was defined as $p<0·05$.

Role of the funding source

The funders of the study had no role in the study design, data collection, data analysis, data interpretation, or writing of the report.

Results

Since November 2018, a large outbreak of NDM-CPE emerged in Tuscany, Italy, predominantly in the northwest of the region.[8] In this area, the incidence of bloodstream infections caused by NDM-CPE exhibited a large increase in 2019 and 2020, and an increasing proportion of bloodstream infections caused by CRE was caused by NDM-CPE during the same period (appendix 1 p 6). The overall 30-day mortality associated with bloodstream infections caused by NDM-CPE observed in 2019–20 was 39·7% (95% CI 33·3–46·4).

Genomic analysis of 54 non-replicated NDM-CPE from bloodstream infections observed in 11 hospitals during the early phases of the outbreak (between Nov 1, 2018, and July 5, 2019) and in the preceding months (between Jan 1, 2018, and Oct 31, 2018), corresponding to 54 (90%) of 60 of all bloodstream infections caused by NDM-CPE reported to the Regional Health Agency of Tuscany in the study period (between Jan 1, 2018, and July 5, 2019), revealed a predominance (48 [89%] of 54 isolates) of NDM-1-producing ST147 *K pneumoniae*, which was clearly associated with the emergence of the outbreak. The remaining isolates included one (2%) NDM-1-producing ST39 *K pneumoniae*, one (2%) NDM-5-producing ST307 *K pneumoniae*, two (4%) NDM-5-producing ST2 *E coli*, and two (4%) NDM-5-producing ST8 *E coli* (figure 1A).

Evaluation of clonal relatedness by core-genome SNPs revealed that the 48 NDM-1-producing ST147 *K pneumoniae* were overall highly related (SNPs range 0–35; mean 12 [SD 6]; median 11 [IQR 8–15]), and clustered in two closely related clades on a maximum-likelihood tree. Clade A (4–19; 13 [7]; 15 [7–18]), encompassed only three isolates and included the earliest available NDM-Kp isolate that was collected in July, 2018 (ie, KP-1PI). Clade B (0–23; 11 [4], 11 [8–13]) included all the remaining isolates (figure 1B).

The 48 NDM-1-producing ST147 *K pneumoniae* from the Tuscan outbreak showed uniform resistance to expanded-spectrum cephalosporins, carbapenems, aztreonam, fluoroquinolones, and the novel β-lactamase inhibitor combinations ceftazidime–avibactam and meropenem–vaborbactam, and frequent resistance to aminoglycosides. They were mostly susceptible to fosfomycin and colistin, and uniformly susceptible to cefiderocol and the aztreonam–avibactam combination (table 1, appendix 2 p 1).

	MIC_{50}	MIC_{90}	Susceptibility (%)	MIC range
Amoxicillin–clavulanic acid*	>64	>64	0	>64
Piperacillin–tazobactam†	>128	>128	0	>128
Ceftazidime	>64	>64	0	>64
Ceftazidime–avibactam	>32	>32	0	32 to >32
Ceftriaxone	>4	>4	0	>4
Cefepime	>16	>16	0	>16
Cefiderocol	1	1	48 (100%)	≤0·25 to 2
Ceftolozane–tazobactam‡	>64	>64	0	>64
Ertapenem	2	>2	0	1 to >2
Meropenem	64	64	1 (2%)	4 to >64
Meropenem–vaborbactam§	64	64	1 (2%)	4 to >64
Aztreonam	>32	>32	0	>32
Aztreonam–avibactam¶	0·5	1	48 (100%)‖	≤0·25 to 1
Amikacin	>32	>32	5 (10%)	≤2 to >32
Gentamicin	>8	>8	5 (10%)	≤0·5 to >8
Ciprofloxacin	>1	>1	0	0·5 to >1
Levofloxacin	>8	>8	0	>8
Colistin	≤0·5	1	45 (94%)	≤0·5 to >8
Fosfomycin	16	128	5 (10%)	4 to >128
Nitrofurantoin	>64	>64	0	>64
Tigecycline	0·5	1	41 (85%)**	≤0·25 to 2
Trimethoprim–sulfamethoxazole††	>8/152	>8/152	1 (2%)	1/19 to >8/512

MIC=minimum inhibitory concentration. *Clavulanic acid at fixed concentration of 2 mg/L. †Tazobactam at fixed concentration of 4 mg/L. ‡Tazobactam at fixed concentration of 4 mg/L. §Vaborbactam at a fixed concentration of 8 mg/L. ¶Avibactam at a fixed concentration of 4 mg/L. ‖Interpreted according to AZT EUCAST clinical breakpoint. **Interpreted according to EUCAST PK-PD (not species-related) breakpoints. ††For the trimethoprim-sulfamethoxazole combinations, different concentration of the partner antibiotic sulfamethoxazole is used and therefore specified (eg, nn/152, nn/19).

Table 1: **Antimicrobial susceptibility profiles of NDM-1-producing ST147 *Klebsiella pneumoniae* isolates (n=48)**

Resistome profiling revealed the constant presence of the chromosomal bla_{SHV-11}, *fosA*, and *oqxA-oqxB* genes, and of a gene encoding a truncated OmpK35. Occasionally, mutations in chromosomal genes associated with colistin resistance (*pmrA, crrB, mgrB*) were observed in 3 (6%) of 48 isolates[13–15] and tigecycline resistance (*ramR, ramA*) were observed in 6 (13%) of 48 isolates (appendix 2 p 2).[16] Concerning the acquired resistome, along with the bla_{NDM-1} and $bla_{CTX-M-15}$ β-lactamase genes, detected in all isolates, and the *armA* gene for 16S rRNA methylase, detected in most (47 [98%] of 48 isolates) of them, a plethora of other acquired determinants associated with resistance to β-lactam antibiotics (bla_{TEM-1}, bla_{TEM-32}, bla_{OXA-1}, and bla_{OXA-9}), aminoglycosides (*aacA4, aadA1, aph[3']-Ia,* and *aph[3']-VI*), sulfonamides (*sul1,* and *sul2*), trimethoprim (*dfrA5*), quinolones (*qnrS1*), rifampicin (*arr-3*), macrolides

See Online for appendix 2

	Size (bp)	Replicons	Plasmidome profile	Resistance or virulence traits	GenBank accession number	Transfer frequencies (transconjugants or donors)
Reference Plasmids						
pQil-NDM-147Tu	56 064	FIB(pQil)	I, II, III, IV	*aacA4-cr, aph(3′)-VI, arr-3, bla*$_{CTX-M-15}$, *bla*$_{NDM-1}$, *ble*$_{MBL}$, *qnrS1, bla*$_{OXA-1}$, *sul1, catB3*	CP071030	..
pR-147Tu.12PI	39 637	R	II, III, IV, V	*aacA4, aadA1, bla*$_{OXA-9}$, *bla*$_{TEM-1}$, *aacA4-cr, aph(3′)-VI, arr-3, bla*$_{CTX-M-15}$, *bla*$_{NDM-1}$, *ble*$_{MBL}$, *qnrS1*	CP072922	..
pR-147Tu.17PI	39 771	R	II, III, IV, V	Same as pR-147Tu.12PI	JAFHLI000000000	..
pR-147Tu.26PI	39 771	R	II, III, IV, V	Same as pR-147Tu.12PI	CP072929	..
pR-147Tu.7PI	38 606	R	II, III, IV, V	Same as pR-147Tu.12PI	JAFHLG000000000	..
pRQ-147Tu.135LU	42 041	R	II, III, IV, V	Same as pR-147Tu.12PI	CP070893	..
pVIR-147Tu.1PI	340 567	HIB-FIB(Mar)	I, II, III, IV, V	*(aph(3′)-Ia)*, armA, bla*$_{CTX-M-15}$, *dfrA5, mph(A), mph(E), msr(E), sul1, sul2, iucABCD-iutA, rmpADC, rmpA2*†, *luxR, peg-344, shiF, cobW, terABCDEFWXYZ*‡	CP071028	$1{\cdot}7\times10^{-5}$
pVIR-147Tu.12PI	341 914	HIB-FIB(Mar)	I, II, III, IV, V	Same as pVIR-147Tu.12PI	CP072918	$1{\cdot}6\times10^{-5}$
pVIR-147Tu.17PI	342 037	HIB-FIB(Mar)	I, II, III, IV, V	Same as pVIR-147Tu.12PI	JAFHLI000000000	$1{\cdot}4\times10^{-4}$
pVIR-147Tu.26PI	318 691	HIB-FIB(Mar)	I, II, III, IV, V	Same as pVIR-147Tu.12PI	CP072926	$1{\cdot}7\times10^{-5}$
pVIR-147Tu.7PI	341 552	HIB-FIB(Mar)	I, II, III, IV, V	Same as pVIR-147Tu.12PI	JAFHLG000000000	$4{\cdot}6\times10^{-5}$
pVIR-147Tu.135LU	341 276	HIB-FIB(Mar)	I, II, III, IV, V	Same as pVIR-147Tu.12PI	CP070891	$1{\cdot}7\times10^{-3}$
pX4-147Tu.17PI	37 887	X4	III	*bla*$_{TEM-32}$	JAFHLI000000000	..
pFIIK-FIB-147Tu.12PI	81 589	FII$_{K1}$-FIB	II	..	CP072920	..
pColE1-147Tu	9293	ColE-like	II, IV	..	CP072923	..
pColE2-147Tu	4167	ColE-like	I, II, III, IV, V	..	CP071031	..
Bacteriophages						
vB_Kpn_147Tu	112 868	..	I, II, III, IV, V	..	CP071029	..
vB_Kpn_147Tu.26PI	114 192	..	IV	..	CP072927	..

*Not detected in pVIR-ST147-Tu.135LU and pVIR-ST147-Tu.26PI. †A frameshift mutation was observed in *rmpA2* in pK2044 and pVIR147Tu-like plasmids compared with pLVPK. ‡Not detected in pVIR-ST147-Tu.26PI, which carried only *terF*.

Table 2: **Overview of the accessory genome of representative NDM-1-producing ST147 *Klebsiella pneumoniae* isolates characterised in this study**

(*mph[A], mph[E], msr[E]*), and phenicols (*catB3*) were also variably present (appendix 2 p 2).

Overall, the resistome was consistent with the resistance profile. Four isolates carried the ArmA allelic variant Ile270Lys in the absence of other relevant aminoglycoside resistance determinants, and were susceptible to amikacin and gentamicin, suggesting that this ArmA variant was not expressed or not associated with pan-aminoglycoside resistance (appendix 2 pp 1–2).

In silico typing of plasmid incompatibility groups on draft genomic assemblies of the 48 NDM-1-producing ST147 *K pneumoniae* identified seven different replicons, including X4, R, FIB(pQil), the multireplicons HIB-FIB(Mar) and FII$_{K1}$-FIB, and two ColE-like replicons, variably distributed among the isolates. Based on the distribution of these replicons, five different plasmidome profiles, presented as I to V, were identified (figure 1B, appendix 2 p 2).

A long-read sequencing approach, done with six randomly selected isolates (ie, KP-1PI, KP-12PI, KP-26PI, KP-7PI, KP-17PI, and KP-135LU) representative of the five different plasmidome profiles, allowed us to generate complete circular molecules for all plasmids (table 2). Subsequent alignment of complete plasmid sequences against the draft genomes revealed an overall conserved plasmid architecture among the NDM-1-producing ST147 *K pneumoniae* isolates from the Tuscan outbreak (figure 1B).

The FIB(pQil)-type plasmid, named pQil-NDM-147Tu, carrying the *bla*$_{NDM-1}$ gene together with several additional resistance determinants (eg, *aacA4-cr* and *bla*$_{CTX-M-15}$), was present in 47 (98%) of 48 isolates (figure 1B) and highly conserved (table 2). This plasmid shared part of the backbone (99% identity; 51% coverage) with the pKpQIL FII-FIB plasmid (accession number NC_014016), which has a major role in the worldwide dissemination of KPC-type carbapenemases.[17] In a single isolate (KP-135LU), a large segment of this plasmid, comprising part of the resistance region, had recombined with the R-type element to yield chimeric plasmid pRQ-147Tu (table 2). Attempts to transfer these NDM-1-encoding plasmids by conjugation were unsuccessful (table 2), in agreement with the absence of conjugal transfer genes from their backbones. Similar NDM-encoding elements were not detected in the accessory genome of the few NDM-CPE of other lineages or species isolated in 2018, including the ST39 and ST307 *K pneumoniae* and the ST2 and ST8 *E coli* isolates (figure 1A; appendix 2 p 2), further underscoring their unrelatedness to the outbreak.

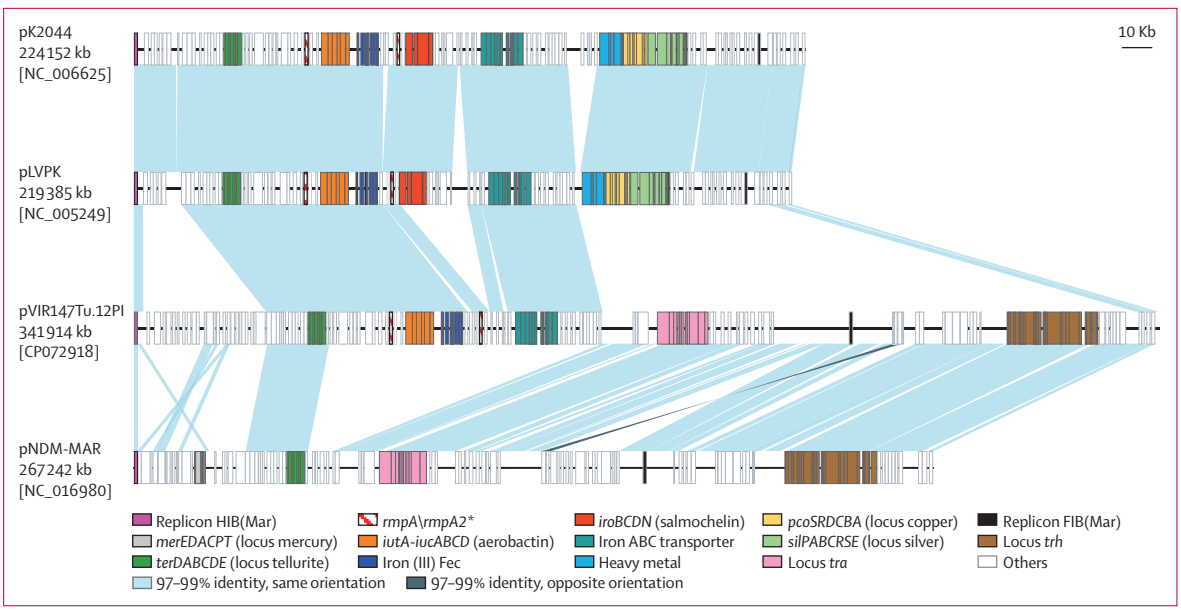

Figure 2: **Comparison of virulence-associated plasmids and closest relatives**
Linear map of plasmids pK2044 and pLVPK (the archetypal virulence elements from the hypervirulent *Klebisella pneumoniae* strains NTUH-K2044 [ST23, serotype K1] and CG43 [ST86, serotype K2]), of plasmid pVIR147Tu from KP-12PI (a representative NDM-1-producing ST147 *K pneumoniae* isolated during the Tuscan outbreak), and of pNDM-MAR (from an NDM-1-producing ST15 *K pneumoniae* from Morocco). Blocks between plasmids indicate a sequence identity spanning 97–99%. Coding DNA sequences are represented as rectangles, and those representing major plasmids' loci are shown with a colour-based legend according to their function. *A frameshift mutation was observed in *rmpA2* in pK2044 and pVIR147Tu from KP-12PI compared with pLVPK.

The large HIB-FIB(Mar) plasmid, named pVIR-147Tu, was present in all the NDM-1-producing ST147 *K pneumoniae* isolates (figure 1B) and carried several determinants of resistance (eg, $bla_{CTX\text{-}M\text{-}15}$, *armA*, *dfrA5*, and *sul1*) and virulence (table 2). This plasmid exhibited an original mosaic structure derived from the recombination between the HIB-FIB resistance plasmid pNDM-MAR (97% identity; >71% coverage), first detected in an ST15 *K pneumoniae* from Morocco,[12] and the virulence plasmids from hypervirulent *K pneumoniae* strains (eg, pK2044 and pLVpK; 99% identity; >50% coverage).[18] As such, pVIR147Tu had a pNDM-MAR-like backbone, including its HIB and FIB replicons and transfer operons (*tra* and *trh*), associated with most of the virulence determinants of pK2044 and pLVpK except for the salmochelin locus (*iroBCDE*/*iroN;* figure 2; appendix 1 p 14). Gene transfer experiments confirmed that the pVIR147Tu plasmid could be transferred by conjugation (table 2), in agreement with the presence of an intact conjugation module. A comparison with publicly available sequences revealed that similar plasmids have also been detected in *K pneumoniae* of ST147 and of different sequence types (ie, ST307, ST11, ST15, ST395, and ST383) that have been reported from various countries over the past 3 years (appendix 1 p 9).[19–23] Of the other plasmids, some carried resistance determinants to various antimicrobials (eg, $bla_{TEM\text{-}32}$), whereas others were not associated with any known resistance or virulence determinant (table 2).

The accessory genome of NDM-1-producing ST147 *K pneumoniae* isolates also included circular prophages belonging to the *Siphoviridae* family (figure 1B), although no association with known resistance or virulence determinants was observed for these elements (table 2).

All the NDM-1-producing ST147 *K pneumoniae* isolates from the Tuscan outbreak carried a large array of acquired virulence determinants, including genes for the siderophores yersiniabactin (*ybtSXQPA, irp1, irp2, ybtUTE,* and *fyuA*) and aerobactin (*iutA-iucABCD*), for regulators of the mucoid phenotype (*rmpADC and rmpA2,* the latter carrying a frameshift mutation), for proteins involved in iron metabolism (*cobW*), for a haemin and lysine transport system (*shiF*), for a putative SAM-dependent methyltransferase or metabolic transporter (*peg-344*), and for a transcriptional regulator-controlling virulence gene expression (*luxR*; appendix 1 p 14).[24] Except for the yersiniabactin-encoding genes, associated with an ICEKp3 chromosomal element, all other virulence determinants were located on the pVIR147Tu plasmid (table 2). Moreover, some isolates also exhibited mutations in chromosomal genes (ie, *pal, csrD,* and *ramR;* appendix 2 p 2) that were previously shown to be involved in modulation of the virulence phenotype.[25–28] Notably, all isolates were negative to string testing.

Experiments in the *G mellonella* infection model were performed using the six isolates representative of different plasmidome profiles, which also exhibited diversity in the *pal, csrD,* and *ramR* genes (appendix 2 p 2), and the *K pneumoniae* NTUH-H2044 hypervirulent strain and the less virulent ST258 *K pneumoniae* KKBO-1 strain

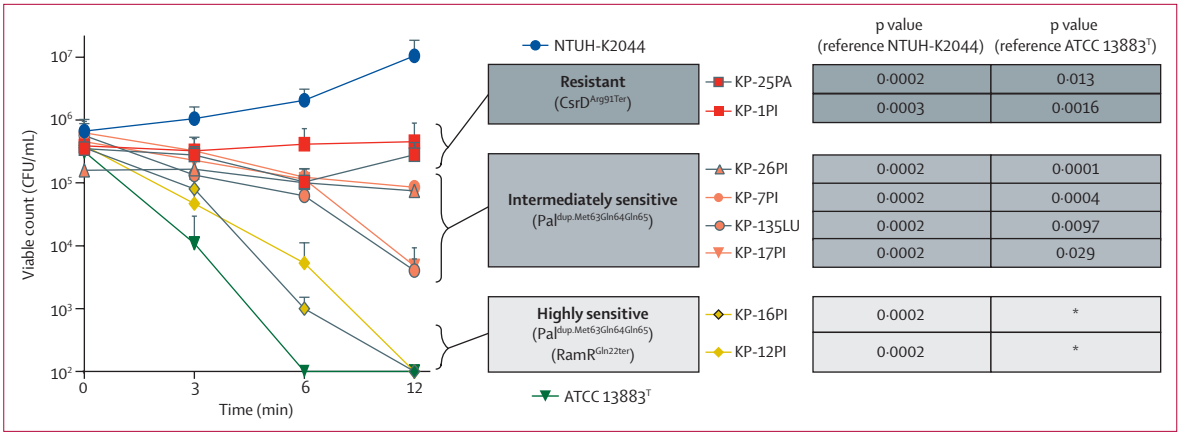

Figure 3: **Evaluation of the bactericidal activity of human serum on representative ST147 *Klebisella pneumoniae* isolates**
Data are mean (SD). The reference *K pneumoniae* strain ATCC 13883^T and the hypervirulent strain NTUH-K2044 (ST23) were used as controls for the susceptible (ATCC 13883^T) and resistant (NTUH-K2044) grade, respectively. Significant differences (two-tailed unpaired *t* test) of viable counts (120 min) against NTUH-K2044 and ATCC 13883^T are shown in the table. CFU=colony forming unit. dup=duplicate. *Unpaired *t* test could not be used because identical viable counts were recorded.

as comparators.[11,29] Results revealed a variable survival rate of *G mellonella* larvae injected with NDM-1-producing ST147 *K pneumoniae* (appendix 1 p 10), with a LD_{50} of $10^{5·5}$–10^7 colony-forming units per larva, which overlapped that of the NTUH-K2044 reference K1 hypervirulent strain and was significantly lower than that of an ST258 *K pneumoniae* KKBO-1 strain producing the KPC-3 carbapenemase (appendix 1 p 11).

In the serum bactericidal assays, the same representative isolates appeared significantly less resistant to killing than the reference hypervirulent strain NTUH-K2044, and more resistant than the reference *K pneumoniae* strain ATCC 13883^T (figure 3). The NDM-1-producing ST147 *K pneumoniae* isolates exhibited three different grades of serum resistance associated with a different status of *pal*, *csrD*, and *ramR* chromosomal genes. In particular, high serum resistance was observed in isolate KP-1PI, carrying a frameshift mutation (Arg91Ter) in CsrD, which promotes degradation of the capsule-regulating small RNA CsrB (acting as positive regulator of capsule synthesis), and the inactivation of CsrD has been associated with increased serum fitness by promoting capsule production and thickness.[25] Intermediate serum resistance was observed in isolates carrying a three amino acid duplication (Met63Gln64Gln65) in Pal, a peptidoglycan-associated lipoprotein that has a crucial role in maintaining cell envelope integrity and in conferring protection against serum killing, mutations of which have been previously linked to attenuated virulence.[26] Low serum resistance was observed in isolate KP-12PI carrying the same Pal mutation (duplication of Met63Gln64Gln65) plus a frameshift mutation (Gln122Ter) in RamR, known to result in upregulation of RamA, which is involved in tigecycline resistance[28] and has been associated with low serum survival rates and a decreased capsule polysaccharide production among in vitro selected tigecycline-resistant *K pneumoniae* mutants.[27] The association between these mutations and variable resistance to killing by human serum was confirmed by the behaviour of two additional NDM-1-producing ST147 *K pneumoniae* isolates carrying either RamR with Gln122Ter and Pal with duplicated Met63Gln64Gln65 (KP-16PI) or CsrD with Arg91Ter (KP-25PA; figure 3).

A comparative analysis with a global collection of ST147 *K pneumoniae* genomes from the NCBI databases (n=714; appendix 1 pp 12–13) was performed to provide insights about the genetic relatedness of the Tuscan clone with other members of ST147 and its evolutionary history. The analysis showed heterogeneity in the population structure of ST147, with two major lineages differing by the capsule and O loci: KL10 (*wzi420*) capsular locus and O3 or O3a locus, and KL64 (*wzi64*) capsular locus and O2v1 locus (appendix 1 p 7).

The NDM-1-producing ST147 *K pneumoniae* isolates associated with the Tuscan outbreak belonged to the latter lineage and clustered within a clade, named ST147KL64 clade 1 (SNP range 0–282; mean 39 [SD 32]; median 32 [IQR 18–57]), including clinical isolates reported from several countries (ie, Denmark, Egypt, Germany, Hungary, Italy, Lebanon, Myanmar, Thailand, the UK, and the USA since 2013. Members of this clade exhibited some common features in terms of accessory genome, namely the presence of an ICEKp3 element associated with the *ybt9* yersiniabactin gene cluster and the presence of the NDM-1-encoding pQil-NDM-147Tu-like plasmid (appendix 1 pp 7–8). Within this clade, the Tuscan clone was resolved in a subclade, named ST147-vir, also including NDM-Kp from the UK and Egypt collected in 2018–19 (SNP range 0–48; mean 21 [SD 12]; median 24 [IQR 11–30]). The major difference of subclade ST147-vir from other members of ST147KL64 clade 1 was represented by the presence of the pVIR-147Tu-like plasmid (appendix 1 p 8).

Discussion

Tracking the dissemination of successful epidemic clones of CPE and understanding their evolution is important for monitoring and controlling these resistant pathogens. In this study, we did a genomic characterisation of NDM-CPE involved in the emergence of a large regional outbreak in Tuscany, Italy,[7] which has rapidly compounded the local CPE epidemiology. Results revealed a clonal nature of the outbreak, sustained by an NDM-1-producing *K pneumoniae* of ST147, a recognised high-risk clone associated with hospital-acquired infections worldwide, and mediating the global spread of several resistance determinants including NDM-like metallo-β-lactamase genes.[3,30] Large outbreaks sustained by NDM-1-producing ST147 K pneumoniae have recently been reported also in the USA (2016–19) and Poland (2015–19).[31,32]

Analysis of the population structure of the ST147 isolates associated with emergence of the Tuscan outbreak revealed an overall low diversification, supporting the origin from a common ancestor recently introduced in the region. However, some minor intraclonal diversity was also observed, underscoring the occurrence of different microevolutionary trajectories following clonal expansion.

The clone from the Tuscan outbreak belonged to a distinct subclade of ST147, named ST147-vir, which carries the KL64 (*wzi64*) capsular locus along with an outstanding array of resistance and virulence determinants that had likely been acquired in a stepwise manner during evolution of this subclade. The time of first isolation of members this subclade in Italy (July 2018), the UK (December 2018), and Egypt (2018) suggests emergence around this date. By inspecting data from a large European genomic surveillance study conducted between 2013–14,[33] no ST147 *K pneumoniae* of this subclade were identified, reinforcing the hypothesis of a recent emergence.

Overall, comparative genomic analyses suggested the following hypothetical evolutionary trajectory leading to the emergence of the ST147-vir subclade including the Tuscan clone of NDM-1-producing ST147 *K pneumoniae*: acquisition of the *ybt*-encoding element ICEKpn3 and of a pQil-NDM-147Tu-like plasmid, encoding NDM-1, by an ancestor of the ST147KL64 clade 1 leading to evolution of this clade, and subsequent acquisition of a pVIR-147Tu-like mosaic plasmid by a member of this clade, leading to the subclade ST147-vir. However, the possibility that recombination between a pNDM-MAR-like resistance plasmid and a virulence plasmid typical of hypervirulent *K pneumoniae* (eg, pK2044-like and pLVPK-like plasmids) occurred within a member of the ST147KL64 clade 1 could not be excluded. The detection of pVIR-147Tu-like mosaic plasmids in *K pneumoniae* of ST147 and of other sequence types (ie, ST307, ST11, ST15, ST395, and ST383) since 2017 from different countries (ie, Czech Republic, Egypt, Germany, Russia, and the UK),[19–23] suggests that the actual distribution of these mosaic elements carrying virulence and resistance genes could be wide.

The complex resistome of the Tuscan clone accounted for a very broad resistance profile, leaving only a few treatment options such as cefiderocol, the aztreonam–avibactam combination, and, in most but not all cases, fosfomycin and colistin. Indeed, aztreonam–avibactam combinations (administered as aztreonam plus ceftazidime–avibactam) were often used for treating these infections and shown to be superior to regimens based on older drugs.[34]

Concerning virulome, unlike most other members of the ST147, the Tuscan clone carried several acquired virulence factors, including some of those typical of hypervirulent *K pneumoniae* strains (appendix 1 p 14),[24] although it did not show a typical hypermucoviscous phenotype. In the *G mellonella* infection model, representative isolates showed an overall enhanced virulence potential compared with a representative of the ST258 high-risk clone, with LD_{50} often comparable or lower than those of reference hypervirulent *K pneumoniae* strain NTUH-K2044. However, the *G mellonella* model was shown to not accurately differentiate hypervirulent from less virulent *K pneumoniae* strains.[35] Furthermore, the same isolates displayed a significantly lower resistance to human serum than NTUH-K2044, suggesting a different behaviour from the canonical hypervirulent *K pneumoniae* strains. This finding is overall consistent with an observation that one isolate from the same outbreak did not exhibit a hypervirulent phenotype in a mouse subcutaneous infection model.[36] Moreover, the reported 30-day mortality rate of invasive infections caused by the Tuscan NDM-1-producing ST147 *K pneumoniae* (ie, 39·7%) was lower than that reported for infections by typical hypervirulent *K pneumoniae*,[18] and the clinical presentation of infections caused by members of this clone did not include the most frequent manifestations of hypervirulent *K pneumoniae* infections (ie, endophthalmitis and liver abscess).[9] Altogether, these findings suggest that members of the ST147-vir subclade do not behave as typical hypervirulent *K pneumoniae*. This finding could be related with the absence of some acquired virulence determinants (eg, salmochelin encoding or allantoinase-encoding loci) and/or with the different capsular type that could have a pivotal role in establishing a virulence phenotype, as largely documented for the K1, K2, K5, and K57 loci.[18]

A diverse grade of serum resistance was observed with different isolates, possibly associated with the status of some chromosomal genes (*csrD*, *pal*, and *ramR*), which could affect virulence by altering the status or function of bacterial surface components,[25–27,37] revealing the possibility of intraclonal modulation of virulence during clonal expansion. Similarly, the description of two outbreak isolates producing NDM-9 in 2020, and resistant to colistin and fosfomycin, further underscored the remarkable plasticity of this outbreak clone.[38]

Although this study provided novel insights about the phenotypic and genotypic characteristics of the ST147 clone associated with the Tuscan outbreak, it also presented some limitations that should be acknowledged, including not having an assessment of the different virulence grades observed in the serum bactericidal assays using a suitable animal model, the absence of characterisation of isolates representative of different types of infections (along with bloodstream infections) to further understand the diversity and evolution of this clone, and the impossibility to infer the putative emergence of this clone through dated phylogeny because there is a very poor correlation between sampling dates and the genetic distances among sequenced isolates.

The results of this study provide further evidence for the plasticity and the evolutionary potential of high-risk clones of *K pneumoniae*. The convergence of clinically relevant resistance determinants (including metallo-β-lacatmase genes) and virulence determinants typical of hypervirulent *K pneumoniae* strains in chimeric elements like pVIR-ST147Tu, capable of horizontal transfer, could enable the evolution of resistance and virulence among high-risk clones of *Klebsiella*, which is worrisome. Continued efforts in tracking the dissemination of these lineages and characterising their features are therefore warranted to prevent and control their spread in the health-care system.

The Tuscan Clinical Microbiology Laboratory Network

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Contributors

VDP, TG, AA, and GMR conceptualised the study and validated the data. VDP, LHDA, NA, IB, CN, and SF analysed the data. VDP and LHDA wrote the original report and VDP, TG, AA, and GMR reviewed and edited the report. LHDA, NA, IB, CN, SF, TG, and AA collected the data. LR, MTM, TG, and AA supervised the study. All authors had full access to all of the data in the study and the final responsibility to submit for publication. VDP, TG, and AA have accessed and verified all the data in the study.

Declaration of interests

GMR reports grants, personal fees, and non-financial support from Accelerate Diagnostics, personal fees from Becton Dickinson, Zambon, Roche, Thermo Fisher, QPex, Qiagen, and Pfizer, grants and personal fees from bioMérieux, Cepheid, MSD, Shionogi, Beckman Coulter, Menarini, Angelini Pharma, and Nordic Pharma, grants from Seegene, Arrow, Symcel, Hain Lifescience, Meridian, SetLance, Qvella, Qlinea, Biomedical Service, Quidel, and DID, and personal fees and fees for bacterial strains from Venatorx, outside the submitted work. TG reports personal fees from Alifax, bioMérieux, Thermo Fisher Scientific, Seegene, Accelerate Diagnostics, and VenatorX, and grants from AstraZeneca, outside the submitted work. AA reports personal fees from Seegene, Menarini, Arrow Diagnostic, and Accelerate Diagnostic, and non-financial support from SymCel, outside the submitted work. IB reports non-financial support from Diesse Diagnostica Senese, outside the submitted work. All other authors declare no competing interests.

Data sharing

Raw Illumina reads and complete and draft genomes of the sequenced New Delhi metallo-β-lactamases carbapenemase-producing Enterobacterales have been deposited in the National Center for Biotechnology Information databases under the BioProject PRJNA643814.

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