

In vivo evolution to hypermucoviscosity and ceftazidime/avibactam resistance in a liver abscess caused by *Klebsiella pneumoniae* sequence type 512

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ABSTRACT Carbapenemase-producing *Klebsiella pneumoniae* represents a major public health issue globally. Isolates with resistance to the newest drugs, like ceftazidime/avibactam (CZA), are increasingly reported. In this study, we analyzed the evolution of KPC-3-producing sequence type (ST) 512 *K. pneumoniae* strains isolated at three different times (hospitalization days 45, 56, and 78) from the same patient, two of which were observed in a pericholecystic liver abscess. The three *K. pneumoniae* isolates (295Kp, 304Kp, and hmv-318Kp) from the same patient were subjected to antimicrobial susceptibility testing, whole-genome sequencing, sedimentation assay, biofilm measurement, serum resistance assay, macrophage phagocytosis, and adhesion assays. KPC-producing isolate hmv-318Kp exhibited carbapenem susceptibility, hypermucoviscous (hmv) colony phenotype and CZA resistance. Virulence markers of hypervirulent *Klebsiella* were absent. Two non-synonymous mutations were identified in the hmv-318Kp genome comparing with isogenic strains: a single-nucleotide polymorphism (SNP) occurred in the pKpQIL plasmid, changing *bla*_{KPC-3} in the *bla*_{KPC-31} gene variant, conferring CZA resistance; and a second SNP occurred in the *wzc* gene of the capsular biosynthesis cluster, encoding a tyrosine kinase, resulting in the F557S Wzc protein mutation. The *Klebsiella pneumoniae* strain exhibiting an hmv phenotype (hmv-Kp) phenotype has been previously associated with amino acid substitutions occurring in the Wzc tyrosin kinase protein. We observed *in vivo* evolution of the ST512 strain to CZA resistance and acquisition of hypermucoviscosity. The pathogenetic role of the detected Wzc substitution is not fully elucidated, but other Wzc mutations were previously reported in hmv *K. pneumoniae*. Wzc mutants may be more frequent than expected and an underreported cause of hypermucoviscosity in *K. pneumoniae* clinical isolates.

IMPORTANCE Here we describe the evolution of KPC-3-producing ST512 *K. pneumoniae* isolated at three different times from the same patient of which the last one, from a biliary abscess, showed CZA resistance by KPC-31 production and manifested hmv colony phenotype. Hypervirulent *Klebsiella pneumoniae* (hv-Kp) isolates are increasingly reported worldwide. Their hypervirulent traits are associated with the presence of *rmpA/A2* genes and an hmv. In this study, we identified an hmv-Kp that lacked the *rmpA-D* cluster but showed an amino acid substitution in the Wzc tyrosin kinase protein, involved in the capsular biosynthesis. This hmv-Kp strain emerged *in vivo* and evolved resistance to ceftazidime/avibactam resistance in a liver abscess of a patient. Our findings suggest that *wzc* mutations may be underreported, making it challenging to distinguish hv-Kp from “classic” *K. pneumoniae* with an hmv phenotype.

Editor Raffaele Zarrilli, University of Napoli Federico II, Naples, Italy

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Valerio Capitani and Gabriele Arcari contributed equally to this article. The first author first identified the isolates under study.

The authors declare no conflict of interest.

See the funding table on p. 9.

Parts of the data presented in this article were presented as abstracts and posters at the 34th ECCMID Congress held in Barcelona, Spain, on 27–30 April 2024.

Received 20 May 2024

Accepted 15 July 2024

Published 22 August 2024

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KEYWORDS *wzc*, capsule, serum susceptibility, ST512, KPC-31, antibiotic resistance, hmv, hypervirulence, string test, *rmpA* negative, mucoviscous, hypermucoid

The *Klebsiella pneumoniae* clonal group (CG) 258 is endemic in several countries and often associated with the production of the *Klebsiella pneumoniae* carbapenemase. In Italy, most the *K. pneumoniae* strains can be assigned to the CG258 (47.4%) lineage, with sequence type (ST) 512 representing the most common clone (1). The KPC class A serine-carbapenemase confers resistance to most beta-lactams, including carbapenems (2). In 2016, the European Medicines Agency authorized the clinical use of ceftazidime/avibactam (CZA), a combination of a third-generation cephalosporin (ceftazidime) with a new-generation non-beta-lactam, beta-lactamase inhibitor (avibactam), to treat serine-carbapenemase-producing *Enterobacterales* (3).

Despite the great activity against KPC, CZA resistance is an emerging issue and is mostly due to the production of KPC variants, with increased ceftazidimase activity and reduced affinity for the avibactam inhibitor (4, 5).

Recent studies have identified a *Klebsiella pneumoniae* strain exhibiting an hmv phenotype (hmv-Kp) (6–8) which is unrelated to the so-called “hypervirulent” pathotype (hvKp) (9). Hypervirulent strains typically feature a virulence plasmid that includes the *rmpA-D* and *iucABCD-iutA* clusters, which contributes to an enhanced virulence phenotype (10). Unlike hvKp, hmv-Kp lacks the *rmpA-D* genes associated with capsular cluster upregulation and the other virulence determinants (11). The genetic determinants and pathogenic potential of hmv-Kp remain largely unclear (12).

In most of the studies, the hmv-Kp phenotype has been associated with amino acid substitutions occurring in the Wzc tyrosin kinase protein, which is one of the major factors responsible for capsule export and polymerization (6, 7, 13, 14). It has been predicted that the autophosphorylation process plays a central role in the Wzc function (15).

The expression of virulence traits like the accessory genes responsible for the hypermucoviscous phenotype in ST512 isolates is anecdotal (16), and no ST512 genome in the Institut Pasteur database (<https://bigsd.b.pasteur.fr/klebsiella/>) carries the associated genes (i.e., *rmpA-D*).

Here we describe an ST512 *K. pneumoniae* strain showing CZA resistance by KPC-31 production and manifested hypermucoviscous (hmv) colony phenotype by Wzc mutation.

RESULTS

Case report and phenotypic description of the *Klebsiella pneumoniae* isolates

In January 2022, a patient was transferred to the coronavirus disease 2019 intensive care unit (ICU) of the Policlinico Umberto I (PUI) in Rome, Italy, from another hospital. On day 35 of hospitalization, the patient tested negative for severe acute respiratory syndrome coronavirus 2 and was transferred to the general ICU of the PUI.

On day 45, after 10 days in the general ICU, during a routine gut colonization screening, the patient tested positive for carbapenem-resistant KPC-producing *K. pneumoniae* (isolate 295Kp). On day 49, the patient developed a bloodstream infection caused by a carbapenem-resistant KPC-producing *K. pneumoniae* (isolate not available), concurrent with a clinical and radiological picture of acute complicated cholecystitis with pericholecystitis, as shown by an abdominal CT scan. Empiric antibiotic treatment with fosfomicin, tigecycline, and CZA was started. An ultrasound check on day 56 revealed the presence of a pericholecystic liver abscess, and the patient consequently underwent an emergency cholecystostomy. The procedure was performed under ultrasound guidance using a transhepatic approach. Under fluoroscopic guidance, intrahepatic abscess near the gallbladder bed was drained. The gallbladder was punctured, and an additional cholecystostomy drainage catheter was inserted, with its distal end positioned in the lumen of the gallbladder. The drained material was sent for culture, and the infection was found to be monomicrobial. Carbapenem-resistant KPC-producing

K. pneumoniae was isolated (isolate 304Kp). Clinically, the patient responded to the antibiotic treatment with fosfomycin, tigecycline, and CZA, resulting in fever resolution and progressive clinical improvement. Twenty-two days later (day 78), a carbapenem-susceptible, CZA-resistant KPC-producing *K. pneumoniae* was isolated from biliary drainage; it showed hypermucoviscous colony phenotype (isolate hmv-318Kp). The treatment with fosfomycin, tigecycline, and CZA was continued until day 88. Given the clinical improvement despite the microbiological isolation, the patient was discharged from the ICU to the transplant surgery ward. On day 90, considering the patient's asymptomatic condition, the drainage was removed after imaging confirmation of the resolution of the abscess and cholecystitis. Throughout the 90 days of the patient's hospitalization, the three *K. pneumoniae* isolates (295Kp, 304Kp, and hmv-318Kp), sampled from different sources or exhibiting diverse phenotypes, were subjected to comprehensive analysis for deeper genomics and functional characterization (Table 1; Tables S1 and S2).

Comparative genomic analyses among the three isolates

By genomic analyses, the three isolates were assigned to ST512 by *K. pneumoniae* multilocus sequence typing (MLST) (11) and were negative for the presence of the *rmpA-D* genes, lacking all genetic determinants associated with the typical virulence plasmid causing the hypervirulent *Klebsiella pneumoniae* (hv-Kp) phenotype. The only siderophore present in all the three genomes is yersiniabactin, and by Kleborate tool, the resulting virulence score is 1 (17).

A total of nine single-nucleotide polymorphism (SNPs) were detected on the chromosome sequence, comparing the initial isolate 295Kp from the rectal swab and the first 304Kp isolate from the liver abscess: two caused synonymous substitutions; five caused missense mutations in various metabolic proteins; and one caused a frameshift mutation in a galactosidase protein (Table S1). A recombination event was identified in 304Kp pKpQIL with respect to 295Kp pKpQIL, involving the region of the transfer locus. One SNP (synonymous mutation) was identified on the 304Kp pKpQIL, in the *traN* gene.

All differences identified between 295Kp and 304Kp were also confirmed in hmv-318Kp. Only three additional SNPs were found, comparing 304Kp and the hmv-318Kp the strains isolated from the liver abscess at 22 days of distance. One SNP was identified in the pKpQIL located *bla*_{KPC-3} gene, defining the *bla*_{KPC-31} variant conferring CZA resistance, another one was identified in the chromosomal gene encoding the tyrosine kinase Wzc defining the F557S variant.

A third SNP was identified in the intergenic region adjacent to the repressor of biofilm formation gene *bssS*. This mutation involved neither the -35 nor the -10 promoter sequences of the gene. No differences in the number or position of insertion sequences were observed.

Analyses of the hypermucoviscous phenotype

Out of the three strains under examination, only hmv-318Kp tested positive to the string test (>2.0 cm in length). The hmv-318Kp strain showed higher sedimentation assay

TABLE 1 Characteristics of the *Klebsiella pneumoniae* sequence type 512 strains analyzed in this study

Isolate	Source ^a	Day ^b	Minimal inhibitory concentration KPC		KPC	hmv ^d	Wzc
			(mg/L) ^c				
			CZA	MEM			
295Kp	RS	45	0.5	>8	KPC-3	Negative	WT
304Kp	BA	56	0.19	>8	KPC-3	Negative	WT
hmv-318Kp	BA	78	12	2	KPC-31	Positive	F557S

^aAbbreviations of the strain samples: RS, rectal swab; BA, biliary abscess.

^bDay of isolation of the strain considering day 1 as the first day of hospitalization.

^cCZA, ceftazidime/avibactam; MEM, meropenem.

^dHypermucoviscosity was measured by string test.

values. Sedimentation assay mean values determined for isolates 295Kp and 304Kp were very similar to each other, being $OD_{600} = 0.077$ and $OD_{600} = 0.089$, respectively, while the mean value for the hmv-318Kp isolate was almost four times higher, being $OD_{600} = 0.293$ (Fig. 1A).

The hmv-318Kp strain showed increased susceptibility to human serum, with a mortality rate two logarithms higher after 1 h of exposure in human serum, further increasing after 2 h (Fig. 1B).

Isolates 295Kp and 304Kp exhibited a modest ability to form biofilm, while hmv-318Kp displayed a significantly greater ability to form biofilm, when compared to the D4C *Escherichia coli* strain, used as a positive control (Fig. 1C).

Testing the ability to adhere to bladder epithelial cells, hmv-318Kp exhibited lower adherence than 295Kp and 304Kp and was almost completely removable from the epithelium after 3 h. However, all three *Klebsiella* isolates exhibited lower adhesion ability compared to the adhesive strain of *E. coli* CFT073, which served as a positive control (Fig. 1D). In the human macrophage evasion test, 295Kp and 304Kp strains were almost completely internalized by macrophages (Fig. 2), while hmv-318Kp exhibited a 10-fold reduction of internalization, suggesting that the hypemucoviscous strain has improved ability to evade phagocytosis by macrophages.

DISCUSSION

In this article, we describe the *in vivo* evolution of colonizing and infectious isolates of the ST512 *K. pneumoniae* strain isolated from a single patient. In particular, comparing the two isolates from the liver abscess, we identified only two SNPs located in non-coding regions, one in *bla_{KPC-3}*, becoming the *bla_{KPC-31}* gene variant able to confer CZA resistance (4), and the second in the *wzc* gene, encoding the protein-tyrosine kinase involved in exopolysaccharide and capsular polysaccharide biosynthesis and transport (13). The Wzc F557S variant is the most likely cause of the hmv-colony phenotype observed in the isolate hmv-318Kp.

In *K. pneumoniae*, the hmv phenotype is canonically associated with the *rmpA*-D locus (for regulator of mucoid phenotype A). RmpA plays the role of enhancer of the capsular polysaccharide biosynthesis, by cooperation with RcsB transacting factor in modulating the regulatory activity. RmpD is responsible for the hv-Kp phenotype interacting with the protein Wzc (18). However, the hmv phenotype is not necessarily linked to capsule overexpression (19). Wzc mutations have been previously described to be responsible for the hmv phenotype without other hypervirulence-related determinants (14, 19). Mutations in the *wzc* gene have been previously associated with hmv phenotypes in ST258 in the USA (7) and in ST307 and ST15 in China (6) but occurred in different aminoacidic residues. Actually no *wzc* nucleotide sequence in the National Center for Biotechnology Information databank is the same as the one reported here (Wzc F557S). All these previous examples were sporadic isolates from different strain collections, and the functional role in pathogenesis for *wzc*-mutants still is not clearly defined.

Our patient case allows to open some hypotheses about the properties of the emerged mutant and how it survived. It is possible that in the liver abscess, the isolate was not accessible to sufficient doses of antimicrobials that, along the time, selected the conversion of *bla_{KPC-3}* in the CZA resistant variant *bla_{KPC-31}*. Furthermore, we measured for the hmv phenotype a better evasion of phagocytosis by macrophages that potentially confer an advantage in the abscess context. This analysis confirmed previous reports describing a better ability of *wzc* mutants to evade this type of host defense. The hmv-318Kp has a lower ability to survive in serum when compared with the other non-hmv isogenic isolates. This strain lacked the capacity to adhere to bladder epithelial cells. This cell type was tested to verify the known effects of the Wzc mutations as described by Ernst and colleagues (7). Therefore, phenotypic characteristics of the hmv-318Kp conform to previous observations (8) but undermine the hypothesis of functional Wzc mutations as a factor favoring the bloodstream spread of *K. pneumoniae* (7). Strangely,

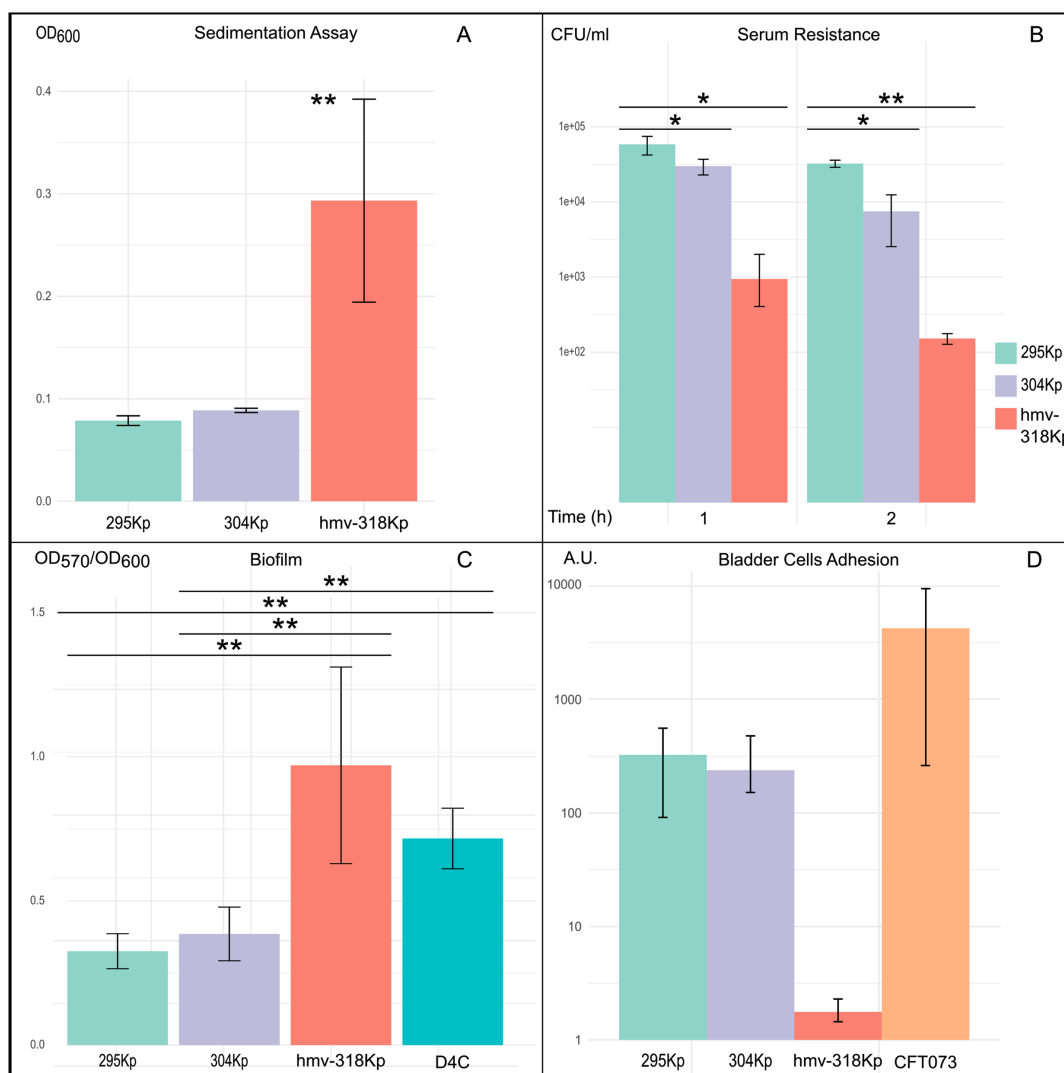


FIG 1 Phenotypic characterization of *Klebsiella pneumoniae* sequence type 512 analyzed in this study. (A) Quantitative sedimentation assay. Isolates, grown overnight in brain heart infusion, were normalized to OD₆₀₀ = 1.0 centrifuged at 1,000 × g for 5 min, and the OD₆₀₀ of the upper 900 μL was measured. Data are shown as means + SDs (n = 6). (B) Serum resistance assay. Tolerance to human sera pool was evaluated by exposing bacteria (5 × 10⁵ CFU/mL) to 100% human serum and incubating at 37°C for 2 h. CFU/mL were enumerated at T0, after 1 and 2 h. Data are shown as means + SDs (n = 6). (C) Quantitative assessment of biofilm formation. Biofilm formation was measured after 24-h incubation under static conditions and reported as the OD₅₇₀/OD₆₀₀ ratio to normalize the amount of biofilm formed to the total bacterial content. Data are shown as means + SDs (n = 48). (D) Adhesion assay to human bladder epithelial cells. Confluent HTB-9 cell monolayers were infected with each isolate using a multiplicity of infection of 100. Cell surface-adherent bacteria were enumerated after 3-h incubation. The uropathogenic *Escherichia coli* strain CFT073 was included as a positive control. Data are shown as means + SDs (n = 9) and expressed as percentage considering arbitrarily strain 295Kp as the reference. Asterisks above bars represent P values evaluated by one-way analysis of variance: *P < 0.05, **P < 0.01.

we observed that hmv-318Kp has a greater ability to form biofilm. These data partially contrast with previous research, despite employing similar measurement methods (7).

A limitation of this article is that we were unable to identify the specific cause of the enhanced biofilm formation ability of hmv-318Kp. This may be due to the mutation identified upstream of the *bssS* gene, although it does not involve the -10 and -35 promoter sequences.

Our description also opens the hypothesis that *wzc* mutations are probably underreported in “classic” *Klebsiella* showing the hypemucoviscous phenotype. Increasing numbers of reports are describing hypemucoviscosity in isolates lacking the *rmpA-D* genes, but the determinants associated with this phenotype were not identified (20–25).

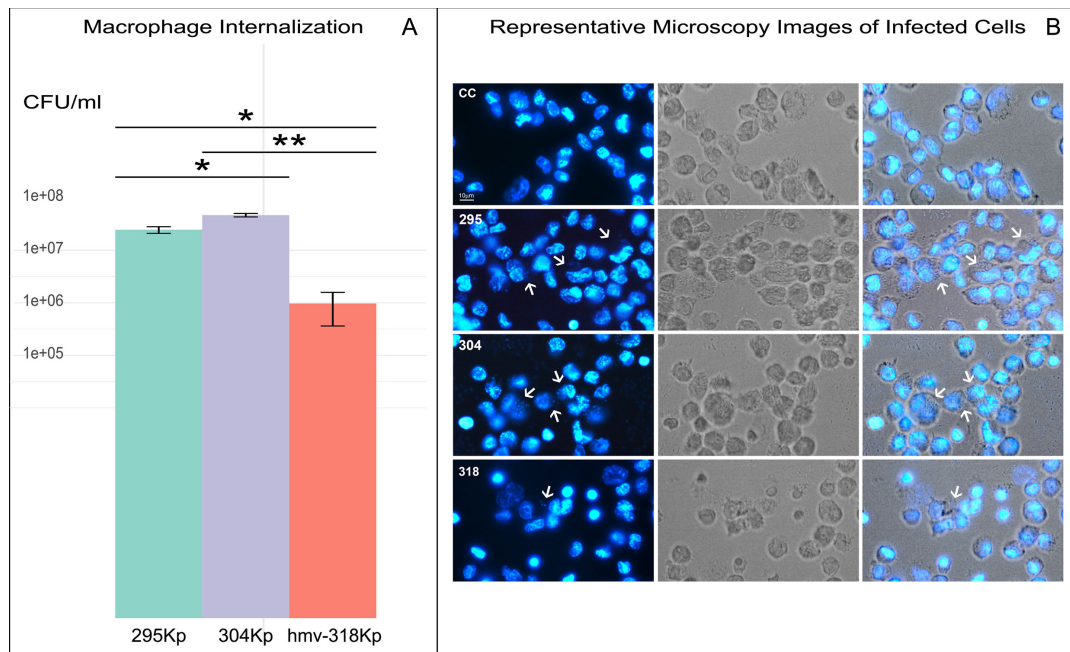


FIG 2 Evasion from phagocytosis by human macrophages of *Klebsiella pneumoniae* sequence type 512 strains isolated in this study. (A) Macrophage internalization assay. Confluent THP-1 cell monolayers were infected with each isolate using a multiplicity of infection of 100. Two hours after infection, chloramphenicol at 60 $\mu\text{g}/\text{mL}$ was added to the medium to kill extracellular bacteria. Internalized bacteria were enumerated after an additional 1 h of incubation. Data are shown as means + SDs ($n = 9$). Asterisks above bars represent P values evaluated by one-way analysis of variance: * $P < 0.05$, ** $P < 0.01$. (B) Representative microscopy images of infected cells. THP-1 cell monolayers infected in parallel were acquired in bright and fluorescence fields 4',6-diamidino-2-phenylindole (DAPI). Scale bar = 10 μm .

This implies that effectively recognizing hv-Kp from classic *K. pneumoniae* with hmv phenotype represents an even more complex challenge than expected.

MATERIALS AND METHODS

Isolation, identification, and collection of *K. pneumoniae* strains

Bacteria were isolated during routine diagnostic procedures from the intensive care unit of the Policlinico Umberto I University Hospital.

Species identification was performed by matrix-assisted laser desorption ionization–time of flight mass spectrometry system (Bruker Daltonik GmbH, Bremen, Germany), and antimicrobial susceptibility testing was performed by the Vitek2 system (bioMérieux, Marcy-l'Étoile, France). Fosfomicin MIC was confirmed using the gold-standard agar dilution method (Liofilchem, Roseto degli Abruzzi, Italy), and CZA MIC was confirmed using gradient strips (Liofilchem).

All “ESKAPE” pathogens (26) isolates sampled from the PUI ICU are stored for epidemiological purposes. To collect the isolates, those are grown on brain heart infusion (BHI) agar plates (Oxoid Limited, Basingstoke Hampshire, United Kingdom), incubated overnight at 37°C, resuspended in glycerol 10% (wt/wt) Luria-Bertani broth, and stored at –80°C.

Three prototypical isolates (listed in order of sampling: 295Kp from rectal swab, 304Kp and hmv-318Kp from biliary abscess) displaying phenotypic differences were collected and analyzed (Table 1).

String test

K. pneumoniae strains were grown at 37°C overnight on 5% sheep blood agar and MacConkey agar plates. A standard 10- μ L bacteriological loop was used to stretch a single colony, and the formation of a continuous filament >5 mm long was considered as a positive string test (27).

Sedimentation assay

Sedimentation assay was performed in triplicate on the three isolates under examination. The sedimentation assay was adapted from previously described protocols (28). Isolates were grown overnight in BHI at 37°C in agitation at 100 rpm. The overnight cultures were centrifuged at 16,000 $\times g$ for 15 min, the obtained pellet was resuspended at OD₆₀₀ = 1.0 in a final volume of 1 mL phosphate-buffered saline (PBS). Samples were centrifuged at 1,000 $\times g$ for 5 min, and the OD₆₀₀ of the upper 900 μ L was measured.

Serum resistance

Whole blood was collected from two healthy volunteer donors. After 30 min at room temperature (RT), the clot was removed by centrifuging at 1,500 $\times g$ for 10 min in a refrigerated centrifuge. The resulting supernatants, designated as sera, were pooled together. Overnight bacterial cultures were normalized, measuring OD₆₀₀, and 1 mL of each culture was centrifuged at 13,000 $\times g$ for 5 min, washed twice with an equal volume of PBS. Then, 50 μ L of each culture corresponding to 5 $\times 10^5$ CFU/mL for each strain was added to 1 mL of pooled sera, as described by Guo et al. (29). CFUs per milliliter were enumerated at T0, after 1 and 2 h. Three independent experiments were repeated, each carried out in duplicate.

Infection assays

Cultured HTB-9 (ATCC-LGC, Milan, Italy) human bladder epithelial cell line and THP-1 (ATCC TIB-202) human monocytes (differentiated to macrophages with 50 nM of 12-O-tetra-decanoylphorbol-13-acetate for 48 h) were used for infection experiments, as previously described (30). Cell monolayers were cultured in Dulbecco's modified Eagle medium (DMEM) and RPMI 1640, respectively, at 37°C under 5% CO₂ atmosphere until 90% confluency and were infected using a multiplicity of infection of 100. To synchronize the infection, plates were centrifuged at 540 $\times g$ for 10 min. Bladder cells were infected for 3 h, while macrophages were incubated with bacteria for 2 h followed by 1 h in the presence of chloramphenicol at 60 μ g/ml. Cell monolayers were washed five times with PBS to remove unbound/extracellular bacteria and subsequently treated with 1 mL of 0.1% Triton X-100 for 5 min. Following lysis, bacteria were quantified by plating out 10-fold dilutions of the bacterial suspensions. Quantifications were performed in triplicate on 3 different days, and the mean results were expressed as percentage, considering arbitrarily strain 295Kp as the reference. The well-characterized uropathogenic *E. coli* strain CFT073 was included as a positive control for adhesion assays (31).

Biofilm measurement

Biofilm formation was measured using the microtiter plate assay, as previously described (32, 33). Briefly, overnight bacterial cultures were normalized to 1 $\times 10^9$ cells/mL, and 20 μ L was added to 180 μ L of LB. Microplates were incubated for 24 h under static conditions. After washing, biofilms were fixed with methanol and stained with crystal violet (1%) for 15 min at RT. Crystal violet was solubilized with ethanol, and absorbance was measured at 570 nm. Results are reported as the OD₅₇₀/OD₆₀₀ ratio to normalize the amount of biofilm formed to the total bacterial content. Four independent experiments, 12 wells per strain ($n = 48$), were performed.

Whole-genome sequencing and bioinformatic analyses

Whole-genome sequencing was carried out, performing both short- and long-read technologies on every isolate. Specifically, short-read Illumina sequencing (Illumina Inc., San Diego, CA, USA) was performed on genomic DNA purified using the Isolate II genomic DNA Extraction Kit (Bioline, Cincinnati, USA), while long-read Oxford Nanopore Technologies (ONT) sequencing (ONT, Oxford, UK) was performed on high-molecular-weight genomic DNA extracted using the Monarch HMW DNA Extraction Kit for Tissue (New England Biolabs, Massachusetts, USA). Unicycler v.0.4.8.0 (34) was used with standard polishing parameters and a normal bridging mode to combine the Illumina performed by SPAdes (35) and the ONT assembly obtained by Flye (36), and its results were refined by the Bandage tool (37).

The resulting assemblies were typed by PlasmidFinder (38) to assess plasmid content and by Kleborate (11) to detect virulence-associated and anti-microbial resistance (AMR) genes.

SNPs among the three genomes were analyzed using the Snippy tool (<https://github.com/tseemann/snippy>), comparing Unicycler v.0.4.8.0 final sequences and gbk files annotated by Bakta (39). Promoter prediction was performed using Softberry Prediction of Bacterial Promoters (<http://www.softberry.com>). The insertion sequences are identified with ISfinder (40). Recombinant regions were predicted using Gubbins (41), and the annotated genomes were visualized with Proksee (42). When possible, bioinformatic analyses were carried out via the public Galaxy.eu server (<https://usegalaxy.eu>) (43).

ACKNOWLEDGMENTS

This research was supported by European Union (EU) funding within the NextGeneration EU-MUR PNRR Extended Partnership initiative on Emerging Infectious Diseases (project nos. PE00000007 and PE13 INF-ACT to A.T.P.) (spoke 4) and A.C. (spoke 3); Italian Minister of University and Research PRIN 2022 Project 2022FN7ANE to A.C.; Bandi Ateneo Sapienza (grant numbers RP12218162DFBF0A and RP1221816196D9A2); SEED PNR 2021 335/2021 to D.S.; and Italian Ministry of Health (Ricerca corrente) to IRCCS San Raffaele Roma, 00166 Rome, Italy, to C.A. R.P. was supported by the PNRR PHD scholarship (EX M.D. 351/22) financed by the Rome Technopole project. The salary of D.S. was supported by POR Lazio FSE 2014-2020 and Sapienza Ateneo funding.

All authors made substantial contributions to this work and approved the final manuscript. V.C. and G.A. contributed equally. Concept and design: V.C., G.A., A.C., C.A., and D.S.; acquisition, analysis, and interpretation of data: V.C., G.A., C.A., D.S., A.D.F., R.P., and G.R.; writing (original draft): G.A., V.C., and A.C.; curation of the clinical case description: G.C.; writing (review and editing): C.A., D.S., A.D.F., R.P., G.R., A.T.P., M.C., C.M., G.C., and P.V.

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FUNDING

Funder	Grant(s)	Author(s)
Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR)	EU-MUR PNRR PE00000007 PE13 INF-ACT	Anna Teresa Palamara Alessandra Carattoli
Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR)	PRIN 2022 Project 2022FN7ANE	Alessandra Carattoli
Sapienza Università di Roma (Sapienza)	RP12218162DFBF0A and RP1221816196D9A2 and SEED PNR 2021 335/2021	Daniela Scribano
Ministero della Salute (Italy Ministry of Health)	Ricerca corrente]" to IRCCS San Raffaele Roma	Cecilia Ambrosi
Sapienza Università di Roma (Sapienza)	Rome Technopole POR Lazio FSE 2014-2020	Riccardo Polani

AUTHOR CONTRIBUTIONS

Valerio Capitani, Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft | Gabriele Arcari, Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft | Cecilia Ambrosi, Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft | Daniela Scribano, Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft | Mariateresa Ceparano, Data curation, Formal analysis, Methodology, Writing – review and editing | Riccardo Polani, Data curation, Formal analysis, Methodology, Writing – review and editing | Alice De Francesco, Data curation, Formal analysis, Methodology, Writing – review and editing | Giammarco Raponi, Data curation, Formal analysis, Supervision, Writing – review and editing | Giancarlo Ceccarelli, Data curation, Investigation, Writing – review and editing | Paolo Villari, Funding acquisition, Supervision, Writing – review and editing | Anna Teresa Palamara, Funding acquisition, Supervision, Writing – review and editing | Carolina Marzuillo, Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review and editing | Alessandra Carattoli, Conceptualization, Funding acquisition, Project administration, Supervision, Validation, Writing – original draft

DATA AVAILABILITY

Biosamples, Sequence Read Archive, and whole-genome sequencing have been released for the three genomes under study in BioProject ([PRJNA1100498](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1100498)). The complete genomes and plasmid content of isolates 304Kp and hmv-318Kp have been released under accession numbers [JBCPSL000000000](https://www.ncbi.nlm.nih.gov/nuccore/JBCPSL000000000) and [JBCPSK000000000](https://www.ncbi.nlm.nih.gov/nuccore/JBCPSK000000000), respectively.

ETHICS APPROVAL

This work was conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from all volunteers who participated at this study. The institutional ethics board of the Policlinico Umberto I University Hospital of Rome approved this study (protocol no. 0282/2024).

ADDITIONAL FILES

The following material is available [online](#).

Supplemental Material

Table S1 and S2 (mSphere00423-24-S0001.pdf). Genomic and phenotypic differences among the three strains analyzed in this study.

REFERENCES

- Di Pilato V, Errico G, Monaco M, Giani T, Del Grosso M, Antonelli A, David S, Lindh E, Camilli R, Aanensen DM, Rossolini GM, Pantosti A, AR-ISS Laboratory Study Group on carbapenemase-producing *Klebsiella pneumoniae*. 2021. The changing epidemiology of carbapenemase-producing *Klebsiella pneumoniae* in Italy: toward polyclonal evolution with emergence of high-risk lineages. *J Antimicrob Chemother* 76:355–361. <https://doi.org/10.1093/jac/dkaa431>
- Queenan AM, Bush K. 2007. Carbapenemases: the versatile β -lactamases. *Clin Microbiol Rev* 20:440–458. <https://doi.org/10.1128/CMR.00001-07>
- Ehmann DE, Jahić H, Ross PL, Gu R-F, Hu J, Kern G, Walkup GK, Fisher SL. 2012. Avibactam is a covalent, reversible, non- β -lactam β -lactamase inhibitor. *Proceedings of the National Academy of Sciences*. , Vol. 109, p 11663–11668
- Taracila MA, Bethel CR, Hujer AM, Papp-Wallace KM, Barnes MD, Rutter JD, VanPelt J, Shurina BA, van den Akker F, Clancy CJ, Nguyen MH, Cheng S, Shields RK, Page RC, Bonomo RA. 2022. Different conformations revealed by NMR underlie resistance to ceftazidime/avibactam and susceptibility to meropenem and imipenem among D179Y variants of KPC β -lactamase. *Antimicrob Agents Chemother* 66:e02124-21. <https://doi.org/10.1128/aac.02124-21>
- Carattoli A, Arcari G, Bibbolino G, Sacco F, Tomolillo D, Di Lella FM, Trancassini M, Faino L, Venditti M, Antonelli G, Raponi G. 2021. Evolutionary trajectories toward ceftazidime-avibactam resistance in *Klebsiella pneumoniae* clinical isolates. *Antimicrob Agents Chemother* 65:e00574-21. <https://doi.org/10.1128/AAC.00574-21>
- Ali MR, Yang Y, Dai Y, Lu H, He Z, Li Y, Sun B. 2023. Prevalence of multidrug-resistant hypervirulent *Klebsiella pneumoniae* without defined hypervirulent biomarkers in Anhui, China: a new dimension of hypervirulence. *Front Microbiol* 14:1247091. <https://doi.org/10.3389/fmicb.2023.1247091>
- Ernst CM, Braxton JR, Rodriguez-Osorio CA, Zagieboylo AP, Li L, Pironti A, Manson AL, Nair AV, Benson M, Cummins K, Clatworthy AE, Earl AM, Cosimi LA, Hung DT. 2020. Adaptive evolution of virulence and persistence in carbapenem-resistant *Klebsiella pneumoniae*. *Nat Med* 26:705–711. <https://doi.org/10.1038/s41591-020-0825-4>
- Zaborskytė G, Hjort K, Lytsy B, Sandegren L. 2024. Convergent within-host evolution alters key virulence factors in a *Klebsiella pneumoniae* clone during a large hospital outbreak. *Microbiology*. <https://doi.org/10.1101/2024.02.06.577356>
- Shon AS, Bajwa RPS, Russo TA. 2013. Hypervirulent (hypermucoviscous) *Klebsiella pneumoniae*: a new and dangerous breed. *Virulence* 4:107–118. <https://doi.org/10.4161/viru.22718>
- Lan P, Jiang Y, Zhou J, Yu Y. 2021. A global perspective on the convergence of hypervirulence and carbapenem resistance in *Klebsiella pneumoniae*. *J Glob Antimicrob Resist* 25:26–34. <https://doi.org/10.1016/j.jgar.2021.02.020>
- Lam MMC, Wick RR, Watts SC, Cerdeira LT, Wyres KL, Holt KE. 2021. A genomic surveillance framework and genotyping tool for *Klebsiella pneumoniae* and its related species complex. *Nat Commun* 12:4188. <https://doi.org/10.1038/s41467-021-24448-3>
- Walker KA, Miner TA, Palacios M, Trzilova D, Frederick DR, Broberg CA, Sepúlveda VE, Quinn JD, Miller VL. 2019. A *Klebsiella pneumoniae* regulatory mutant has reduced capsule expression but retains hypermucoviscosity. *mBio* 10:e00089-19. <https://doi.org/10.1128/mBio.00089-19>
- Yang Y, Liu J, Clarke BR, Seidel L, Bolla JR, Ward PN, Zhang P, Robinson CV, Whitfield C, Naismith JH. 2021. The molecular basis of regulation of bacterial capsule assembly by Wzc. *Nat Commun* 12:4349. <https://doi.org/10.1038/s41467-021-24652-1>
- Khadka S, Ring B, Krzeminski LR, Hathaway M, Walker RS, Mobley HLT, Mike LA. 2022. Regulation of *Klebsiella pneumoniae* mucoidy by the bacterial tyrosine kinase Wzc. *Microbiology*. <https://doi.org/10.1101/2022.06.05.494587>
- Vincent C, Duclos B, Grangeasse C, Vaganay E, Riberty M, Cozzone AJ, Doublet P. 2000. Relationship between exopolysaccharide production and protein-tyrosine phosphorylation in Gram-negative bacteria. *J Mol Biol* 304:311–321. <https://doi.org/10.1006/jmbi.2000.4217>
- Shelenkov A, Mikhaylova Y, Voskanyan S, Egorova A, Akimkin V. 2023. Whole-genome sequencing revealed the fusion plasmids capable of transmission and acquisition of both antimicrobial resistance and hypervirulence determinants in multidrug-resistant *Klebsiella pneumoniae* isolates. *Microorganisms* 11:1314. <https://doi.org/10.3390/microorganisms11051314>
- Argimón S, David S, Underwood A, Abrudan M, Wheeler NE, Kekre M, Abudahah K, Yeats CA, Goater R, Taylor B, et al. 2021. Rapid genomic characterization and global surveillance of *Klebsiella* using pathogen-watch. *Clin Infect Dis* 73:S325–S335. <https://doi.org/10.1093/cid/ciab784>
- Ovchinnikova OG, Treat LP, Teelucksingh T, Clarke BR, Miner TA, Whitfield C, Walker KA, Miller VL. 2023. Hypermucoviscosity regulator RmpD interacts with Wzc and controls capsular polysaccharide chain length. *mBio* 14:e0080023. <https://doi.org/10.1128/mbio.00800-23>
- Walker KA, Treat LP, Sepúlveda VE, Miller VL. 2020. The small protein RmpD drives hypermucoviscosity in *Klebsiella pneumoniae*. *mBio* 11:e01750-20. <https://doi.org/10.1128/mBio.01750-20>
- Altayb HN, Elbadawi HS, Baothman O, Kazmi I, Alzahrani FA, Nadeem MS, Hosawi S, Chaieb K. 2022. Genomic analysis of multidrug-resistant hypervirulent (Hypermucoviscous) *Klebsiella pneumoniae* strain lacking the hypermucoviscous regulators (rmpA/rmpA2). *Antibiotics* 11:596. <https://doi.org/10.3390/antibiotics11050596>
- Dey T, Chakraborty A, Kapoor A, Warrier A, Nag VL, Sivashanmugam K, Shankar M. 2022. Unusual hypermucoviscous clinical isolate of *Klebsiella pneumoniae* with no known determinants of hypermucoviscosity. *Microbiol Spectr* 10:e0039322. <https://doi.org/10.1128/spectrum.00393-22>
- Wozniak JE, Band VI, Conley AB, Rishishwar L, Burd EM, Satola SW, Hardy DJ, Tsay R, Farley MM, Jacob JT, Dumyati G, Jordan IK, Weiss DS. 2019. A nationwide screen of carbapenem-resistant *Klebsiella pneumoniae* reveals an isolate with enhanced virulence and clinically undetected colistin heteroresistance. *Antimicrob Agents Chemother* 63:00107–00119. <https://doi.org/10.1128/AAC.00107-19>

23. Arena F, Menchinelli G, Di Pilato V, Torelli R, Antonelli A, Henrici De Angelis L, Coppi M, Sanguinetti M, Rossolini GM. 2022. Resistance and virulence features of hypermucoviscous *Klebsiella pneumoniae* from bloodstream infections: results of a nationwide Italian surveillance study. *Front Microbiol* 13:983294. <https://doi.org/10.3389/fmicb.2022.983294>
24. Merla C, Kuka A, Mileto I, Petazzoni G, Gaiarsa S, De Vitis D, Ardizzone M, Corbella M, Baldanti F, Cambieri P. 2024. One-year surveillance for hypervirulent *Klebsiella pneumoniae* detected carbapenem-resistant superbugs. *Microbiol Spectr* 12:e0329223. <https://doi.org/10.1128/spectrum.03292-23>
25. Wahl A, Fischer MA, Klaper K, Müller A, Borgmann S, Friesen J, Hunfeld K-P, Ilmberger A, Kolbe-Busch S, Kresken M, Lippmann N, Lübbert C, Marschner M, Neumann B, Pfennigwerth N, Probst-Keppler M, Rödel J, Schulze MH, Zautner AE, Werner G, Pfeifer Y. 2024. Presence of hypervirulence-associated determinants in *Klebsiella pneumoniae* from hospitalised patients in Germany. *Int J Med Microbiol* 314:151601. <https://doi.org/10.1016/j.ijmm.2024.151601>
26. Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, Scheld M, Spellberg B, Bartlett J. 2009. Bad bugs, no drugs: no ESKAPE! An update from the infectious diseases society of America. *Clin Infect Dis* 48:1–12. <https://doi.org/10.1086/595011>
27. Fang C-T, Chuang Y-P, Shun C-T, Chang S-C, Wang J-T. 2004. A novel virulence gene in *Klebsiella pneumoniae* strains causing primary liver abscess and septic metastatic complications. *J Exp Med* 199:697–705. <https://doi.org/10.1084/jem.20030857>
28. Mike LA, Stark AJ, Forsyth VS, Vornhagen J, Smith SN, Bachman MA, Mobley HLT. 2021. A systematic analysis of hypermucoviscosity and capsule reveals distinct and overlapping genes that impact *Klebsiella pneumoniae* fitness. *PLOS Pathog* 17:e1009376. <https://doi.org/10.1371/journal.ppat.1009376>
29. Guo H, Wu Y, Li L, Wang J, Xu J, He F. 2023. Global emergence of carbapenem-resistant *Klebsiella pneumoniae* co-carrying multiple carbapenemases. *Comput Struct Biotechnol J* 21:3557–3563. <https://doi.org/10.1016/j.csbj.2023.07.013>
30. Scribano D, Sarshar M, Prezioso C, Lucarelli M, Angeloni A, Zagaglia C, Palamara AT, Ambrosi C. 2020. d-mannose treatment neither affects uropathogenic *Escherichia coli* properties nor induces stable FimH modifications. *Molecules* 25:316. <https://doi.org/10.3390/molecules25020316>
31. Sarshar M, Scribano D, Limongi D, Zagaglia C, Palamara AT, Ambrosi C. 2022. Adaptive strategies of uropathogenic *Escherichia coli* CFT073: from growth in lab media to virulence during host cell adhesion. *Int Microbiol* 25:481–494. <https://doi.org/10.1007/s10123-022-00235-y>
32. Ambrosi C, Sarshar M, Aprea MR, Pompilio A, Di Bonaventura G, Strati F, Pronio A, Nicoletti M, Zagaglia C, Palamara AT, Scribano D. 2019. Colonic adenoma-associated *Escherichia coli* express specific phenotypes. *Microbes Infect* 21:305–312. <https://doi.org/10.1016/j.micinf.2019.02.001>
33. Ashwath P, Deekshit VK, Rohit A, Dhinakaran I, Karunasagar I, Karunasagar I, Akhila DS. 2022. Biofilm formation and associated gene expression in multidrug-resistant *Klebsiella pneumoniae* isolated from clinical specimens. *Curr Microbiol* 79:73. <https://doi.org/10.1007/s00284-022-02766-z>
34. Wick RR, Judd LM, Gorrie CL, Holt KE. 2017. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput Biol* 13:e1005595. <https://doi.org/10.1371/journal.pcbi.1005595>
35. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol* 19:455–477. <https://doi.org/10.1089/cmb.2012.0021>
36. Lin Y, Yuan J, Kolmogorov M, Shen MW, Chaisson M, Pevzner PA. 2016. Assembly of long error-prone reads using de Bruijn graphs. *Proc Natl Acad Sci U S A* 113:E8396–E8405. <https://doi.org/10.1073/pnas.1604560113>
37. Wick RR, Schultz MB, Zobel J, Holt KE. 2015. Bandage: interactive visualization of *de novo* genome assemblies. *Bioinformatics* 31:3350–3352. <https://doi.org/10.1093/bioinformatics/btv383>
38. Carattoli A, Zankari E, García-Fernández A, Voldby Larsen M, Lund O, Villa L, Møller Aarestrup F, Hasman H. 2014. *In silico* detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. *Antimicrob Agents Chemother* 58:3895–3903. <https://doi.org/10.1128/AAC.02412-14>
39. Schwengers O, Jelonek L, Dieckmann MA, Beyvers S, Blom J, Goesmann A. 2021. Bakta: rapid and standardized annotation of bacterial genomes via alignment-free sequence identification. *Microb Genom* 7:11. <https://doi.org/10.1099/mgen.0.000685>
40. Siguier P, Perochon J, Lestrade L, Mahillon J, Chandler M. 2006. ISfinder: the reference centre for bacterial insertion sequences. *Nucleic Acids Res* 34:D32–6. <https://doi.org/10.1093/nar/gkj014>
41. Croucher NJ, Page AJ, Connor TR, Delaney AJ, Keane JA, Bentley SD, Parkhill J, Harris SR. 2015. Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. *Nucleic Acids Res* 43:e15. <https://doi.org/10.1093/nar/gku1196>
42. Grant JR, Enns E, Marinier E, Mandal A, Herman EK, Chen C-Y, Graham M, Van Domselaar G, Stothard P. 2023. Proksee: in-depth characterization and visualization of bacterial genomes. *Nucleic Acids Res* 51:W484–W492. <https://doi.org/10.1093/nar/gkad326>
43. Afgan E, Baker D, Batut B, van den Beek M, Bouvier D, Cech M, Chilton J, Clements D, Coraor N, Grüning BA, Guerler A, Hillman-Jackson J, Hiltmann S, Jalili V, Rasche H, Soranzo N, Goecks J, Taylor J, Nekrutenko A, Blankenberg D. 2018. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. *Nucleic Acids Res* 46:W537–W544. <https://doi.org/10.1093/nar/gky379>