Whole genome sequencing characterization of Slovenian carbapenem-resistant *Klebsiella pneumoniae*, including OXA-48 and NDM-1 producing outbreak isolates

Katarina Benulič*1, Mateja Pirš1, Natacha Couto2, Monika Chlebowicz2, John W. A. Rossen2, Tomaž Mark Zorec1, Katja Seme1, Mario Poljak1, Tatjana Lejko Zupanc3, Eva Ružič-Sabljić1, Tjaša Cerar1

1 Faculty of Medicine, Institute of Microbiology and Immunology, University of Ljubljana, Ljubljana, Slovenia, 2 Department of Medical Microbiology and Infection Prevention, University Medical Center Groningen, University of Groningen, Groningen, Netherlands, 3 Department of Infectious Diseases, University Medical Center Ljubljana, Ljubljana, Slovenia

* katarina.ben@gmail.com

Abstract

Objectives
The first hospital outbreak of carbapenemase-producing Enterobacteriaceae in Slovenia occurred in 2014–2016. Whole genome sequencing was used to analyse the population of carbapenem-resistant *Klebsiella pneumoniae* collected in Slovenia in 2014–2017, including OXA-48 and/or NDM-1 producing strains from the outbreak.

Methods
A total of 32 *K. pneumoniae* isolates were analysed using short-read sequencing. Multi-locus sequence typing and core genome multi-locus sequence typing were used to infer genetic relatedness. Antimicrobial resistance markers, virulence factors, plasmid content and *wzi* types were determined. Long-read sequencing was used for six isolates for detailed analysis of plasmids and their possible transmission.

Results
Overall, we detected 10 different sequence types (STs), the most common being ST437 (40.6%). Isolates from the initial outbreak belonged to ST437 (12/16) and ST147 (4/16). A second outbreak of four ST15 isolates was discovered. A new ST (ST3390) and two new *wzi* types (*wzi*-556, *wzi*-559) were identified. *bla*OXA-48 was found in 17 (53.1%) isolates, *bla*NDM-1 in five (15.6%), and a combination of *bla*OXA-48/NDM-1 in seven (21.9%) isolates. Identical plasmids carrying *bla*OXA-48 were found in outbreak isolates sequenced with long-read sequencing technology.
Conclusions

Whole genome sequencing of Slovenian carbapenem-resistant *K. pneumoniae* isolates revealed multiple clusters of STs, two of which were involved in the first hospital outbreak of carbapenem producing *K. pneumoniae* in Slovenia. Transmission of the plasmid carrying *bla*OXA-48 between two STs was likely to have occurred. A previously unidentified second outbreak was also discovered, highlighting the importance of whole genome sequencing in detection and/or characterization of hospital outbreaks and surveillance of drug-resistant bacterial clones.

Introduction

Antimicrobial resistance is a matter of concern worldwide and carbapenemase-producing Enterobacteriaceae (CPE) pose a major threat to human health. Resistance to carbapenems can be caused by production of carbapenemases (KPC, NDM, OXA-48, VIM), spreading mainly due to acquired plasmids or other mobile elements, and permeability alterations caused by the loss of porins and overexpressed efflux system [1,2].

In recent years, increased resistance to carbapenems in *Klebsiella pneumoniae* has been reported in several European countries, with variable distribution of predominant types of carbapenemases [1,3,4]. The most common *K. pneumoniae* clonal groups (CGs), as defined by multi-locus sequence typing (MLST), which are associated with outbreaks, are CG258 (sequence type (ST) 258 and its derivatives, including ST11, ST340 and ST437), CG14/15, CG17/20, CG43 (including ST101) and ST147 [5]. A recent study shows that the most common clonal lineages in Europe are indeed ST11, ST15, ST101, ST258 and their derivatives [4], and also an emerging high-risk clone ST307 [6,7]. High-risk clones ST258, ST14, ST37, ST147 and ST101 are associated with carbapenemase resistance, while ST15 and ST17 usually carry extended spectrum beta-lactamases [8].

In Slovenia, a small Central European country with 2 million inhabitants, systematic laboratory surveillance of carbapenem-resistant (CR) Enterobacteriaceae began in the second half of 2010. Until late 2014, only sporadic cases of CPE were detected (up to 10 patients with CPE per year), isolated mainly from surveillance samples. Colonization and/or infection with such strains was most frequently associated with previous hospitalization abroad, notably Serbia [9]. Slovenia lies at the eastern border of Italy and northern border of the Balkans, where the epidemiological situation has been worsening for years, with most countries reporting at least sporadic hospital outbreaks and two countries reporting interregional spread [10,11]. The worst affected country in the Balkans is Serbia with high incidence of CR-*K. pneumoniae* [10], mainly NDM-1 and OXA-48 producers [3,12]. Italy is also an endemic country with predominately KPC carbapenemases [3,4,11], which has recently also experienced a significant NDM outbreak [13].

In Slovenia, as elsewhere, *K. pneumoniae* was the most frequently isolated CPE species (50%), followed by *Enterobacter* spp. (25%) and *Escherichia coli* (17%) [6,9,14]. During the period 2014–2017, a total of 91 patients with CP-*K. pneumoniae* were identified, almost half of those were part of the first Slovenian hospital outbreak of CPE which began at the end of October 2014 in the largest tertiary teaching hospital, lasting until February 2016. A total of 40 patients were affected: OXA-48- and/or NDM-producing *K. pneumoniae* were isolated from 31 patients, CP-*K. pneumoniae* and CP-*E. coli* producing OXA-48 and/or NDM-1 were
simultaneously present in a further seven patients, and OXA48-producing *E. coli* alone was detected in two. Two patients had also other CPE in combination with CP-*K. pneumoniae*. The outbreak was investigated with classic epidemiological investigation and genotyping of the isolates was performed using pulse-field gel electrophoresis and MLST determination [15].

While data obtained using classical approach can provide a general overview of the situation, data obtained using whole genome sequencing (WGS) gives much more detailed and relatively rapid insight into the situation as WGS not only has much higher discriminatory power than PFGE [16], but also allows simultaneous detection of antimicrobial resistance genes, plasmids and virulence factors. We have thus used WGS to assess the population structure of CR-*K. pneumoniae* between 2014 and 2017, and to further elucidate the first hospital CPE outbreak with OXA-48 and NDM-1 carbapenemases. We analysed the genomes of selected *K. pneumoniae* isolates to determine their relatedness and to detect possible high-risk clones, antimicrobial resistance markers, virulence factors and plasmid content. We used long-read sequencing on selected outbreak isolates to look for horizontal spread of a transmissible plasmid between these isolates.

### Materials and methods

#### Selection of isolates

A total of 32 *K. pneumoniae* isolates were included in this study. Outbreak isolates were selected based on PFGE profiles [15]. The remaining isolates were selected to reflect the Slovenian population of carbapenemases. All were chosen from the laboratory collection of Institute of Microbiology and Immunology in Ljubljana, which serves as the Slovenian national expert laboratory, between 2014 and 2017 (S1 Table). Our selection included 16 CP-*K. pneumoniae* isolates obtained during the hospital outbreak and 16 CP-*K. pneumoniae* isolates unrelated to the outbreak. Six isolates were selected for long-read sequencing, five for the analysis of the *bla*OXA-48 plasmid and one for the confirmation of the *bla*LEN gene (S1 File).

#### Routine bacterial identification and antimicrobial susceptibility testing

MALDI-TOF mass spectrometry (Microflex LT with regularly updated Brucker MS library Brucker Daltonics, Bremen, Germany) was used for identification of organisms. Disk diffusion was used for antimicrobial susceptibility testing according to contemporary EUCAST guidelines ([www.eucast.org](http://www.eucast.org)), 2014–2017, with the ending of the isolate name denoting the year of isolation.

#### Molecular detection of *bla*NDM, *bla*KPC, *bla*OXA-48-like, *bla*IMP and *bla*VIM

Multiplex real-time PCR targeting the genes *bla*NDM, *bla*KPC, *bla*OXA-48-like, *bla*IMP and *bla*VIM was performed using the LightMix Modular Carbapenemase kits (TIB Molbiol, Berlin, Germany) [15].

#### Short-read whole-genome sequencing

QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) was used to extract genomic DNA for short-read WGS. Fourteen libraries were prepared using the Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA). A further 18 libraries were prepared using the Nextera DNA Flex Library Prep Kit (Illumina, San Diego, CA, USA). Sequencing was performed on the Illumina MiSeq Platform (2 x 300 bp). FastQC 0.11.8 ([https://www.bioinformatics.babraham.ac.uk/projects/fastqc/](https://www.bioinformatics.babraham.ac.uk/projects/fastqc/)) was used for quality control of raw reads. Default settings for
trimming were used in Ridom SeqSphere 5.1.0. (Ridom, GmbH, Munster, Germany). Briefly, trimming was performed on both ends of the reads until the average base quality was > 30 in a window of 20 bases. Reads were subsequently assembled de novo using the Velvet assembly tool in Ridom SeqSphere 5.1.0. (Ridom GmbH, Muenster, Germany) with default settings [17]. Assembly quality check was performed by Ridom SeqSphere and Quast programme [18] (S1 Table).

**Long-read whole genome sequencing using Oxford nanopore technologies**

DNA for long-read sequencing by Oxford Nanopore Technologies (ONT, Oxford, UK) was isolated using the PureLink Genomic DNA Mini Kit (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA), following the manufacturer’s instructions. Libraries were prepared using Native Barcoding (EXP-NBD104) and Ligation Sequencing Kits (SQK-LSK109) (ONT). DNA products were sequenced in a GridION X5 system (ONT) on a FLO-MIN106 flow cell for 48 h. Basecalling was conducted using Guppy v2.0.5 (ONT), and Porechop v0.2.3_seqan2.1.1 was used for sequence trimming and to demultiplex the dataset (https://github.com/rrwick/Porechop). Original Illumina reads were trimmed with Trimomatic v0.39 [19]. Hybrid de novo sequence assemblies were obtained using Unicycler v0.4.7 [20].

**MLST genotyping, wzi typing, virulence genes, resistance genes and plasmid detection**

MLST [21], wzi typing and detection of genes encoding virulence factors were achieved by uploading contigs to the Pasteur Institute website (http://bigsdb.pasteur.fr) [22–24]. Virulence score was calculated according to Kleborate software (https://github.com/katholt/Kleborate). Determination of resistance genes, detection of plasmids and pMLST were performed with default settings using ResFinder 3.1 [25], PlasmidFinder 2.0 and pMLST 1.4 (https://cge.cbs.dtu.dk/services/) [26], respectively. New ST and wzi types were submitted to the Pasteur Institute website, where new identification numbers were assigned. Six isolates sequenced using ONT were also analysed using ResFinder 3.1 [25], PlasmidFinder 2.0 and pMLST 2.0 [26]. The Bandage programme was used for identification of carbapenemase genes [27]. Plasmids of five isolates positive for blaOXA-48, which were sequenced using ONT, and a reference plasmid from the Kp11978 strain [28,29], were compared and visualized using the BLAST Ring Image Generator (BRIG) [30]. The plasmid was annotated using Geneious 8.1.8 (Biomatters, Ltd., Auckland, New Zealand) and annotations were additionally compared to non-redundant protein sequences database using blastx at NCBI (http://blast.ncbi.nlm.nih.gov). Our five aforementioned isolates were also compared in EasyFig [31].

**Nucleotide sequence accession number**

Sequencing data have been submitted to the European Nucleotide Archive (study accession number PRJEB32863).

**Results**

**Real-time PCR to detect AMR genes**

According to real-time PCR, the genes blaOXA-48-like and blaNDM-1 were the most commonly determined, found in 17/32 (53.1%) and 5/32 (15.6%) isolates, respectively (Table 1). Seven out of thirty-two (21.9%) isolates carried both genes. blaKPC-2 and blaVIM-1 genes were found in one isolate each.
Detection of beta-lactamase genes in WGS data

Overall, we detected 30 different beta-lactam resistance genes using WGS (Table 1, S2 Table). The majority of beta-lactamase genes were rare, with 16 genes being present in one isolate each. The most commonly detected was *bla*CTX-M-15, which was found in 27/32 (84.4%) isolates. In one *K. pneumoniae* isolate, *blaLEN* gene was detected. Carbapenemase genes detected with WGS were concordant with the results of the real-time PCR.

Multi-locus sequence typing

Based on short-read WGS data, we detected 10 different STs (Table 1, Fig 1). The most frequent type was ST437 (13/32; 40.6%). Outbreak isolates belonged to ST437 (12/16) and ST147.

---

Table 1. WGS characterization of *K. pneumoniae* isolates (n = 32), carbapenem-resistance and wzi typing.

| Isolate  | ST | wzi typing | *bla*KPC-2 | *bla*VIM-1 | *bla*NDM-1 | *bla*OXA-48 | *bla*OXA-181 |
|----------|----|------------|------------|------------|------------|-------------|-------------|
| BR318-14 | 15 | 24         | +          |            |            |             |             |
| BM670-16 | 15 | 24         | +          |            |            |             |             |
| BM367-17 | 15 | 24         | +          |            |            |             |             |
| BR402-14 | 15 | 447        | +          |            |            |             |             |
| BM433-16 | 35 | 37         | +          |            |            |             |             |
| BR406-15 | 37 | 83         |            |            | +          |             |             |
| BR4-14   | 101| 137        | +          | +          | +          |             |             |
| BR605-15 | 101| 137        | +          | +          | +          |             |             |
| BR615-16 | 101| 137        | +          | +          | +          |             |             |
| BM679-17 | 101| 137        | +          | +          | +          |             |             |
| BR370-14 | 147| 64         | +          |            |            |             |             |
| BR387-14 | 147| 64         | +          |            |            |             |             |
| BR328-14 | 147| 64         | +          |            |            |             |             |
| BR319-14 | 147| 64         | +          |            |            |             |             |
| BR193-17 | 147| 99         | +          |            |            |             |             |
| BM230-17 | 258| 29         | +          |            |            |             |             |
| BR470-15 | 268| 95         | +          |            |            |             |             |
| BR321-14 | 437| 109        | +          |            |            |             |             |
| BR329-14 | 437| 109        | +          |            |            |             |             |
| BR38-15  | 437| 109        | +          |            |            |             |             |
| BR103-15 | 437| 109        | +          |            |            |             |             |
| BR179-15 | 437| 109        | +          | +          | +          |             |             |
| BR194-15 | 437| 109        | +          | +          | +          |             |             |
| BR211-15 | 437| 109        | +          | +          | +          |             |             |
| BR247-15 | 437| 109        | +          | +          | +          |             |             |
| BR252-15 | 437| 109        | +          | +          | +          |             |             |
| BR254-15 | 437| 109        | +          | +          | +          |             |             |
| BR76-16  | 437| 109        | +          | +          | +          |             |             |
| BR282-16 | 437| 109        | +          | +          | +          |             |             |
| BR207-15 | 437| 109        | +          | +          | +          |             |             |
| BR380-15 | 2384|559   | +          | +          | +          |             |             |
| BR737-16 | 3390|556   | +          | +          | +          |             |             |

* a outbreak isolates
* b additionally determined outbreak isolate

https://doi.org/10.1371/journal.pone.0231503.t001
One of the ST437 isolates (marked in yellow, Fig 1) was initially considered as unrelated to the outbreak according to the epidemiological data, though it clustered together with other ST437 isolates from the outbreak. The four ST147 outbreak isolates did not cluster with the rest of the outbreak isolates and the fifth ST147 isolate was not related to the outbreak. Interestingly, a second outbreak of four closely related isolates of ST15 was discovered. Further four isolates belonged to ST101 but were not closely related to each other. The remaining isolates (6/32) were assigned to different STs, one of which was new, namely ST3390 (gapA:2; infB:1; mdh:1; pgi:1; phoE:361; rpoB:1; tonB:14).

Virulence factors

Complete \textit{mrk} clusters were detected in 24/32 (75.0\%) isolates (S2 Table). All 32 isolates carried \textit{mrkB}, \textit{mrkC}, \textit{mrkF} and \textit{mrkJ}. Complete \textit{ybt} (yersiniabactin) cluster was detected in 5 isolates, for which a virulence score 1 was calculated. Complete \textit{iuc} (aerobactin) locus was detected in four isolates, three of them belonging to the outbreak. Colibactin and salmochelin coding loci were not detected among our isolates. In 27/32 (84.4\%) isolates, including all outbreak isolates, we calculated a virulence score 0. Interestingly, ST437 and ST147 outbreak isolates possessed a maximum of 11 virulence genes, with the majority carrying eight genes.

Wzi typing

Overall, 12 different \textit{wzi} types were identified, two of them being new variants, namely \textit{wzi-556} and \textit{wzi-559}. The most frequently detected was \textit{wzi-109}, which was assigned to all (13/13) ST437 isolates (Table 1). Four of five closely related ST147 isolates shared an identical \textit{wzi-64} type; the fifth isolate was \textit{wzi-99}.

Plasmid content

Plasmid analysis revealed a high diversity of incompatibility (Inc) groups (n = 23) (S2 Table). The most frequent was IncL/M (pOXA-48), detected in 23/32 (71.9\%) isolates. IncL/M (pOXA-48) was not detected in two of 24 \textit{blaOXA-48}-positive isolates. All but one (11/12) NDM-1-positive isolates had detectable IncA/C, although other plasmid groups were also detected, e.g. IncFII(K), IncFIB(K), IncHI1B (S2 Table).

Plasmid analysis of five outbreak isolates sequenced with ONT revealed an almost identical plasmid in the Kp11978 reference and all five isolates (ST437, ST147) with a \textit{blaOXA-48} gene detected (Fig 2). The \textit{blaOXA-48} gene, along with the gene for acetyl CoA carboxylase and transcription regulator \textit{lysR} was embedded between the two IS1999. The only difference between our isolates was present in isolate BR38, which had a \textit{mucAB} region inserted (S1 Fig). In one of the aforementioned five ONT sequenced isolates, \textit{blaNDM-1} carrying plasmid was also detected besides \textit{blaOXA-48} plasmid. In the sixth isolate neither of the two plasmids was detected, nor the \textit{blaLEN} gene, which was detected with Illumina sequencing.

Discussion

The most represented ST in \textit{CP-K. pneumoniae} from this study was ST437, a single locus variant of ST258 from the widely distributed clonal group CG258 [32]. This is due to the first
hospital outbreak with aforementioned ST437 and ST147, that occurred in 2014–2016 [15], with OXA-48- and/or NDM-1-producing *K. pneumoniae*. Almost identical plasmid was confirmed in different outbreak isolates belonging to the two STs suggesting plasmid-mediated spread of *bla*OXA-48 resistance genes. This is in concordance with initial molecular analysis of the outbreak where PFGE demonstrated oligoclonal structure in epidemiologically clearly linked patients [15]. A recent study shows that the most common and high-risk clonal lineages in Europe are ST11, ST15, ST101 and ST258 and their derivatives [4], of which STs 15, 101 and 258 were also detected among our isolates. ST307, the cause of the recent outbreak with

Fig 2. BRIG-generated schematic representation of the plasmid encoding *bla*OXA-48 (marked in blue), sequenced using hybrid assemblies of five *Klebsiella pneumoniae* isolates and a reference plasmid from Kp11978 isolate [28,29].

https://doi.org/10.1371/journal.pone.0231503.g002
OXA-48/NDM-1 producing and colistin-resistant *K. pneumoniae* in Germany [7], was not found among our isolates.

WGS analysis revealed an additional outbreak isolate (BR252-15). Epidemiological investigation did not establish any connection between the patient, from whom this *K. pneumoniae* ST437 was isolated, and the wards where other infected patients were hospitalized, whereas our WGS data suggested that this isolate was part of the same outbreak, clustering together with the rest of ST437 outbreak isolates based on core genome MLST. It was also confirmed that the same plasmid, carrying *bla*OXA-48, was present in the patient’s isolate and other outbreak isolates showing the potential of WGS to enhance conventional epidemiologic investigation and allow a more accurate control of an outbreak [33].

We detected a combination of three different beta-lactamase genes *bla*OXA-48, *bla*NDM-1 and *bla*CTX-M-15 in six ST437 isolates (BR179-15, BR194-15, BR207-15, BR211-15, BR254-15, BR252-15), which to our knowledge is the first described combination of these genes in ST437. Co-existence of genes for CTX-M-15 and OXA-48 has been observed in *K. pneumoniae* isolate from our source patient (BR329-14) as well as in ST437 isolates in Serbia [12], where this patient was previously hospitalized [15]. We did not detect *bla*NDM-1 in *K. pneumoniae* isolates of the source patient, we did however detect it in *E. coli* and *Proteus mirabilis* isolate that were isolated from the same surveillance sample which was presumably the source for *bla*NDM-1 in *K. pneumoniae* outbreak isolates [15].

Five ST147 isolates, a clone associated with KPC, VIM, NDM, OXA-48-like and CTX-M-15 producers and a common ST in Europe, with prevalence in the Mediterranean basin, including North African countries [34,35], were detected. Four of them, closely related to each other according to WGS, were epidemiologically confirmed outbreak isolates and clustered together with only one allelic mismatch.

Moreover, four isolates of ST15, a pandemic clone [36], also detected in our neighbouring countries Croatia, Austria, and Hungary and associated with OXA-48, NDM-1 and CTX-M-15 producers [37–39], were detected in our selection, forming an unexpected outbreak. All four isolates shared the same beta-lactamase resistance pattern; however, additional epidemiological investigations have failed to reveal any clear connection.

Although it is one of the most commonly identified STs around the world and in Europe [4,40], ST258 was found in only one patient in our study which is to be expected as this ST is frequently associated with KPC carbapenemase, which is rare in Slovenia (less than 10% of CP-*K. pneumoniae*, Pirs M, personal communication). ST11, one of the most frequently detected STs in Europe [4,41], remained undetected among our isolates.

In the majority of European *K. pneumoniae* isolates that carried more than one carbapenemase gene, the *bla*OXA-48-like and *bla*NDM-like combination was reported to be the most common [4,7], and it is also the only combination detected in our *K. pneumoniae* isolates.

Although *bla*LEN was observed in ST258 isolate identified as *K. pneumoniae* according to short-read assembly, analysis of the hybrid assembly did not show the gene. However, *K. pneumoniae* belonging to ST258 and carrying *bla*LEN has already been described [42]. This discordance of results could be due to use of the two assemblers, which use different algorithms for assembly: Velvet assembler for the short-reads assembly and SPAdes for the hybrid-assembly [8,43].

It has been previously reported that outbreak isolates can have enhanced virulence potential [44], though our findings suggest that the virulence genes alone were not responsible for the successful spread of our outbreak clones, since they had a virulence score 0 and possessed a maximum of 11 virulence genes, whereas other isolates had a detected maximum of 26 genes, which is in accordance with some other studies [45,46].
A complete mrk cluster, coding type-3 fimbriae [22], was detected in majority of our isolates. We did not further investigate the missing genes in the mrk cluster in some isolates, although it has been reported that they could be interrupted by insertion sequences, which results in impaired detection [47]. A complete locus of an alternative siderophore-coding yer-siniabactin was detected in few isolates, but none of them was an outbreak isolate. The yersiniabactin locus is often found in CP-K. pneumoniae and is strongly associated with isolates from the respiratory tract, including CP ST258 [48]. Our results are in concordance with this association, as all eleven genes of the locus were detected in our ST258 CP isolate. The aerobactin (iuc) locus, which consists of iueA-D and iutA genes, has been known for its connection with virulence, and it was suggested its role in virulence is the most crucial among sidero-phere-coding loci [49]. We detected iuc locus in few of the outbreak isolates, which were without detected ybt locus. All of our outbreak isolates were therefore scored by virulence score 0, including majority of the remaining isolates, so our findings show that successful CP-K. pneumoniae clones present in Slovenia were not very virulent.

All of our isolates belonging to the largest cluster ST437 were assigned wzi-109, a combination that has been previously reported [50]. The wzi gene is a part of the cps locus responsible for synthesis of capsule polysaccharide and associated with virulence and capsular switching, important for escaping the host immune response [22,51]. The gene can thus be used for characterization and typing of K. pneumoniae isolates. Previous studies report a possible exchange of the cps locus, including wzi genes, between K. pneumoniae strains, although it is not clear if horizontal exchange and capsular switching is equally common in all clonal groups and STs [5,51]. Some correlation has been reported between certain wzi types and carbapenem-resistance genes, namely KPC-2, ST258 and wzi-29 [40,47]. wzi-29 was indeed detected in one of our isolates belonging to this well-known clone, which was positive for blaKPC-2. We detected two new wzi types.

IncL/M (pOXA-48), an epidemic plasmid connected with the worldwide dissemination of blaOXA-48 [29], was detected in our blaOXA-48 positive isolates, suggesting it could be responsible for the carbapenem resistance in K. pneumoniae isolates in Slovenian hospital. The genetic environment of blaOXA-48 was consistent with previous work [29]. Our findings regarding IncA/C plasmids could be compatible with Hancock et al. [52], highlighting an association with blaNDM, although this gene can also be associated with a number of other plasmids [53,54].

However, assembly of plasmids is difficult to achieve with short reads generated with Illumina Miseq [55], so further analysis is needed for determination of the plasmid responsible for acquired resistance determinants in our isolates. With long-read sequencing of five outbreak isolates, we confirmed almost exactly the same plasmid present in all five isolates (ST437 and ST147) positive for blaOXA-48, suggesting the plasmid-mediated spread of carbapenem-resistance among different STs and showing the importance of WGS in identifying and characterizing outbreaks.

Conclusions

Whole-genome sequencing of a selection of Slovenian CR-K. pneumoniae isolates revealed multiple clusters of sequence types, of which two were involved in the single hospital outbreak of CP-K. pneumoniae in Slovenia. A further isolate belonging to the outbreak was identified and transmission of the blaOXA-48-carrying plasmid was confirmed, highlighting the importance of WGS in detecting and/or characterizing hospital outbreaks. A previously unidentified outbreak of ST15 isolates was unexpectedly discovered, a finding demonstrating the need for better surveillance of drug-resistant bacterial clones.
Supporting information

S1 Table. Quast and SeqSphere assembly quality report.
(XLS)

S2 Table. *K. pneumoniae* isolates (n = 32) characterization including resistance, plasmid and virulence profiles by WGS.
(XLS)

S1 File. Methods. Six isolates were analysed using ONT GridIon. Four sequenced isolates were part of a confirmed outbreak: two belonged to the main cluster of ST437, the other two were selected as the only representatives of ST147 in order to confirm transmission of a plasmid with the *bla*OXA-48 resistance gene among different clones in the outbreak. One isolate of ST437 was previously classified as unrelated to the outbreak according to epidemiological data but clustered together with the outbreak isolates; therefore, we wanted to confirm it as part of the outbreak. The sixth isolate was sequenced because we detected *bla*LEN in *K. pneumoniae* belonging to ST258 and we wanted to confirm the presence of that gene.
(DOC)

S1 Fig. EasyFig-generated schematic representation of *bla*OXA-48 encoding plasmids detected with long-read whole genome sequencing. Black lines represent plasmids from five *K. pneumoniae* isolates positive for *bla*OXA-48 in PCR and short-read whole-genome sequencing. Coloured bars represent shared parts of genome between plasmids. An insertion was detected in isolate BR38 (white triangular insert in the bottom two coloured bars).
(TIF)

Acknowledgments

We thank the team of curators of the Institut Pasteur MLST and whole-genome MLST databases for curating the data and making them publicly available at [http://bigsdb.pasteur.fr/](http://bigsdb.pasteur.fr/).

Author Contributions

**Conceptualization:** Katarina Benulič, Mateja Pirš, Eva Ružič-Šabljić, Tjaša Cerar.

**Data curation:** Katarina Benulič, Mateja Pirš.

**Formal analysis:** Katarina Benulič, John W. A. Rossen, Tjaša Cerar.

**Investigation:** Katarina Benulič, Tjaša Cerar.

**Methodology:** Katarina Benulič, Mateja Pirš, John W. A. Rossen, Tjaša Cerar.

**Project administration:** Katarina Benulič.

**Resources:** Mateja Pirš.

**Software:** Katarina Benulič, Natacha Couto, Monika Chlebowicz, Tomaž Mark Zorec, Tjaša Cerar.

**Supervision:** Eva Ružič-Šabljić, Tjaša Cerar.

**Validation:** Katarina Benulič, Mateja Pirš, Tjaša Cerar.

**Visualization:** Katarina Benulič.

**Writing – original draft:** Katarina Benulič.
Writing – review & editing: Katarina Benulič, Mateja Pirš, Natacha Couto, Monika Chlebowicz, John W. A. Rossen, Tomaz Mark Zorec, Katja Seme, Mario Poljak, Tatjana Lejko Zupec, Eva Ružič-Sabljić, Tjaša Cerar.

References

1. European Centre for Disease Prevention and Control. Surveillance of antimicrobial resistance in Europe, Annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net) 2017. ECDC: Stockholm, Sweden, 2018. Available from: https://www.ecdc.europa.eu/en/publications-data/surveillance-antimicrobial-resistance-europe-2017

2. Pitout JD, Nordmann P, Poirel L. Carbapenemase-producing *Klebsiella pneumoniae*, a key pathogen set for global nosocomial dominance. Antimicrob Agents Chemother. 2015; 59: 5873–5884. https://doi.org/10.1128/AAC.01019-15 PMID: 26169461

3. Grundmann H, Gnaser C, Albiger B, Aanensen DM, Tomlinson CT, Andrašević AT, et al. Occurrence of carbapenemase-producing *Klebsiella pneumoniae* and *Escherichia coli* in the European survey of carbapenemase-producing Enterobacteriaceae (EuSCAPE): a prospective, multinational study. Lancet Infect Dis. 2017; 17: 153–163. https://doi.org/10.1016/S1473-3099(16)30257-2 PMID: 27866944

4. David S, Reuter S, Harris SR, Glaser C, Feltwell T, Argimon S, et al. Epidemic of carbapenem-resistant *Klebsiella pneumoniae* in Europe is driven by nosocomial spread. Nat Microbiol. 2019; 4: 1919–1929. https://doi.org/10.1038/s41564-019-0492-8 PMID: 31358985

5. Wyres KL, Holt KE. *Klebsiella pneumoniae* population genomics and antimicrobial-resistant clones. Trends Microbiol. 2016; 24: 944–956. https://doi.org/10.1016/j.tim.2016.09.007 PMID: 27742466

6. Wyres KL, Hawkey J, Hetland MAK, Fostervold A, Wick RR, Judd LM, et al. Emergence and rapid global dissemination of CTX-M-15-associated *Klebsiella pneumoniae* strain ST307. J Antimicrob Chemother. 2019; 74: 577–581 https://doi.org/10.1093/jac/dky492 PMID: 30517666

7. European Centre for Disease Prevention and Control. Outbreak of carbapenemase-producing (NDM-1 and OXA-48) colistin-resistant *Klebsiella pneumoniae* ST307, north-east Germany, 2019. ECDC: Stockholm, Sweden, 2019. Available from: https://www.ecdc.europa.eu/en/publications-data/outbreak-Klebsiella-pneumoniae-Germany

8. Navon-Venezia S, Kondratyeva K, Carattoli A. *Klebsiella pneumoniae*: a major worldwide source and shuttle for antibiotic resistance. FEMS Microbiol Rev. 2017; 41: 252–275 https://doi.org/10.1093/femsre/fux013 PMID: 28521338

9. Pirš M, Cerar Kišek T, Ambrožič Avguštin J, Kolman J, Seme K, Mueller–Premru M, et al. [Carbapenem-Producing Enterobacteriaceae], Med Razgl. 2013; 52(Suppl 6): S119–127. Slovene.

10. World Health Organization, Regional Office for Europe. The Central Asian and Eastern European Surveillance of Antimicrobial Resistance (CAES AR). The annual report 2018. Copenhagen, Denmark, 2018. Available from: http://www.euro.who.int/en/health-topics/disease-prevention/antimicrobial-resistance/publications/2018/central-asian-and-eastern-european-surveillance-of-antimicrobial-resistance-annual-report-2018-2018

11. Brolund A, Lagerqvist N, Byfors S, Struelens MJ, Monnet DL, Albiger B, et al. Worsening epidemiological situation of carbapenemase-producing Enterobacteriaceae in Europe, assessment by national experts from 37 countries, July 2018. Euro Surveill. 2019; 24: pii = 1900123.

12. Novović K, Trudić A, Barić S, Vasićević Z, Kojić M, Madić D, et al. Molecular epidemiology of colistin-resistant, carbapenemase-producing *Klebsiella pneumoniae*, Serbia, 2013–2016. Antimicrob Agents Chemother. 2017; 61: pii = 602550–16 https://doi.org/10.1128/AAC.02550-16 PMID: 28242665

13. European Centre for Disease Prevention and Control. Regional outbreak of New Delhi metallo-beta-lactamase producing carbapenem-resistant Enterobacteriaceae, Italy, 2018–2019. ECDC: Stockholm, Sweden; 2019. Available from: https://www.ecdc.europa.eu/en/publications-data/RRA-new-delhi-metallo-beta-lactamase-producing-CRE

14. Pirš M, Cerar Kišek T, Križan Hergouth V, Seme K, Mueller Premru M, Logar M, et al. [The course of the first outbreak caused by carbapenem-producing Enterobacteriaceae in Slovenian hospital]. 6. Likarjev simpozij: bolnišnične okužbe, problematika odpornih bakterij, Sekcija za klinično mikrobiologijo in bolnišnične okužbe. Zbornik povzetkov; 2016 Jun 21; Ljubljana, Slovenija. 2016. Slovene. Available from: http://www.imi.si/strokovna-zdruzenja/strokovna-srecanja/6-likarjev-simpozij

15. Pirš M, Cerar Kišek T, Križan Hergouth V, Seme K, Mueller Premru M, Jeverica S, et al. Successful control of the first OXA-48 and/or NDM carbapenemase-producing *Klebsiella pneumoniae* outbreak in Slovenia 2014–2016. J Hosp Infect. 2019; 101: 142–149. https://doi.org/10.1016/j.jhin.2018.10.022 PMID: 30399389

16. Rossen JWA, Friedrich AW, Moran-Gilad J, ESCMID Study Group for Genomic and Molecular Diagnostics (ESGMD). Practical issues in implementing whole-genome-sequencing in routine diagnostic
21. Diancourt L, Passet V, Verhoef J, Grimont PA, Brisse S. Multilocus sequence typing of Klebsiella pneumoniae nosocomial isolates. J Clin Microbiol. 2005; 43: 4178–4182. https://doi.org/10.1128/JCM.43.8.4178-4182.2005 PMID: 16081970

22. Brisse S, Passet V, Haugaard AB, Babosan A, Kassis-Chikhani N, Struve C, et al. wzi gene sequencing, a rapid method for determination of capsular type for Klebsiella strains. J Clin Microbiol. 2013; 51: 4073–4078. https://doi.org/10.1128/JCM.01924-13 PMID: 24088853

23. Bialek-Davenet S, Criscuolo A, Ailloud F, Passet V, Jones L, Delannoy-Vieillard AS, et al. Genomic characterization of hypervirulent and multidrug-resistant Klebsiella pneumoniae clones. Emerg Infect Dis. 2014; 20: 1812–1820. https://doi.org/10.3201/eid1110.140206 PMID: 25341126

24. Brisse S, Fevre C, Passet V, Issenhuth-Jeannel S, Tournebize R, Diancourt L, et al. Virulent clones of Klebsiella pneumoniae: identification and evolutionary scenario based on genomic and phenotypic characterization. PLOS ONE. 2009; 4: e4982. https://doi.org/10.1371/journal.pone.0004982 PMID: 19319196

25. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, et al. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother. 2012; 67: 2640–2644. https://doi.org/10.1093/jac/dks261 PMID: 22782487

26. Carattoli A, Zankari E, García-Fernández A, Voldby Larsen M, Lund O, Villa L, et al. In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Antimicrob Agents Chemother. 2014; 58: 3895–3903. https://doi.org/10.1128/AAC.02412-14 PMID: 24777092

27. Wick RR, Schultz MB, Zobel J, Holt KE. Bandage: interactive visualization of de novo genome assemblies. Bioinformatics. 2015; 31: 3350–3352. https://doi.org/10.1093/bioinformatics/btv383 PMID: 26099265

28. Poirel L, Bonnin RA, Nordmann P. Genetic features of the widespread plasmid coding for the carbapenemase OXA-48. Antimicrob Agents Chemother. 2012; 56: 559–562. https://doi.org/10.1128/AAC.02898-11 PMID: 22083465

29. Poirel L, Héritier C, Tolún V, Nordmann P. Emergence of oxacillinase-mediated resistance to imipenem in Klebsiella pneumoniae. Antimicrob Agents Chemother. 2004; 48: 15–22. https://doi.org/10.1128/JAC.48.1.15-22.2004 PMID: 14693513

30. Alikhan N-F, Petty NK, Ben Zakour NL, Beatson SA. BLAST ring image generator (BRIG): simple prokaryote genome comparisons. BMC Genomics. 2011; 12: 402. https://doi.org/10.1186/1471-2164-12-402 PMID: 21824423

31. Sullivan MJ, Petty NK, Beatson SA. Easyfig: a genome comparison visualizer. Bioinformatics. 2011; 27: 1009–1010. https://doi.org/10.1093/bioinformatics/btr039 PMID: 21278367

32. Seki LM, Pereira PS, de Souza M da PAH, Conceição Mde S, Marques EA, Porto CO, et al. Molecular epidemiology of KPC-2-producing Klebsiella pneumoniae isolates in Brazil: the predominance of sequence type 437. Diagn Microbiol Infect Dis. 2011; 70: 274–277. https://doi.org/10.1016/j.diagmicrobio.2011.01.006 PMID: 21397425

33. Motro Y, Moran-Gilad J. Next-generation sequencing applications in clinical bacteriology. Biomol Detect Quantif. 2017; 14: 1–6. https://doi.org/10.1016/j.bdq.2017.10.002 PMID: 29255684

34. Protonotariou E, Poulou A, Politi L, Sgouropoulos I, Metallidis S, Kachrimanidou M, et al. Hospital outbreak due to a Klebsiella pneumoniae ST147 clonal strain co-producing KPC-2 and VIM-1 carbapenemases in a tertiary teaching hospital in Northern Greece. Int J Antimicrob Agents. 2018; 52: 331–337. https://doi.org/10.1016/j.ijantimicag.2018.04.004 PMID: 29654892

35. Messaoudi A, Haenni M, Mansour W, Sarais E, Bel Haj Khalifa A, Chaouch C, et al. ST147 NDM-1-producing Klebsiella pneumoniae spread in two Tunisian hospitals. J Antimicrob Chemother. 2017; 72: 315–316. https://doi.org/10.1093/jac/dkw401 PMID: 27659734

microbiology. Clin Microbiol Infect. 2018; 24: 355–360. https://doi.org/10.1016/j.cmi.2017.11.001 PMID: 29117578

17. Zerbino DR, Birney E. Velvet: Algorithms for de novo short read assembly using de Bruijn graphs. Genome Res. 2008; 18: 821–829. https://doi.org/10.1101/gr.074492.107 PMID: 18349386

18. Gurevich A, Saveliev V, Vyahhi N, Tesler G. QUAST: quality assessment tool for genome assemblies, Bioinformatics. 2013; 29: 1072–1075. https://doi.org/10.1093/bioinformatics/btt086 PMID: 23422339

19. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 2014; 30: 2114–2120. https://doi.org/10.1093/bioinformatics/btu170 PMID: 24695404

20. Wick RR, Judd LM, Gorrie CL, Holt KE. Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. PLOS Comput Biol. 2017; 13: e1005595. https://doi.org/10.1371/journal.pcbi.1005595 PMID: 28594827

21. Diancourt L, Passet V, Verhoef J, Grimont PA, Brisse S. Multilocus sequence typing of Klebsiella pneumoniae nosocomial isolates. J Clin Microbiol. 2005; 43: 4178–4182. https://doi.org/10.1128/JCM.43.8.4178-4182.2005 PMID: 16081970
PLOS ONE | https://doi.org/10.1371/journal.pone.0231503 April 13, 2020 14 / 14

36. Lee CR, Lee JH, Park KS, Kim YB, Jeong BC, Lee SH. Global Dissemination of Carbapenemase-Producing Klebsiella pneumoniae: Epidemiology, Genetic Context, Treatment Options, and Detection Methods. Front Microbiol. 2016; 7: 895. https://doi.org/10.3389/fmicb.2016.00895 PMID: 27379038

37. Galler H, Feierl G, Petternel C, Reinthaler FF, Haas D, Grisold AJ, et al. KPC-2 and OXA-48 carbapenemase-harboring Enterobacteriaceae detected in an Austrian wastewater treatment plant. Clin Microbiol Infect. 2014; 20: O132–134. https://doi.org/10.1111/1469-0691.12336 PMID: 24033741

38. Melegh S, Kovács K, Gám T, Nyul A, Patkó B, Tóth A, et al. Emergence of VIM-4 metallo-β-lactamase-producing Klebsiella pneumoniae ST15 clone in the Clinical Centre University of Pécs, Hungary. Clin Microbiol Infect. 2014; 20: O27–29. https://doi.org/10.1111/1469-0691.12293 PMID: 23809141

39. Jelić M, Škrinj I, Bejuk D, Košćak I, Butić I, Gužvinec M, et al. Characterization of isolates associated with emergence of OXA-48-producing Klebsiella pneumoniae in Croatia. Microb Drug Resist. 2018; 24: 973–979. https://doi.org/10.1089/mdr.2017.0168 PMID: 29267137

40. Wright MS, Perez F, Brinkac L, Jacobs MR, Kaye K, Cober E, et al. Population structure of KPC-producing Klebsiella pneumoniae isolates from Midwestern U.S. Hospitals. Antimicrob Agents Chemother. 2014; 58: 4961–4965. https://doi.org/10.1128/AAC.00125-14 PMID: 24913165

41. Jayol A, Poirel L, Dortet L, Nordmann P. National survey of colistin resistance among carbapenemase-producing Klebsiella pneumoniae isolates from Midwestern U.S. Hospitals. Antimicrob Agents Chemother. 2014; 58: 4961–4965. https://doi.org/10.1128/AAC.00125-14 PMID: 24913165

42. Pitt ME, Duc Cao M, Butler MS, Ramu S, Ganeshamoorthy D, Blaskovich MAT, et al. Octapeptin C4 induces less resistance and novel mutations in an epidemic carbapenemase-producing Klebsiella pneumoniae ST258 clinical isolate compared to Polymyxins. J Antimicrob Chemother. 2019; 4: 582–593.

43. Bankevich A, Nurk S, Antipov D, Issenhuth-Jeangean S, Tournebize R, Diancourt L, et al. SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol. 2012; 19: 455–477. https://doi.org/10.1089/cmb.2012.0021 PMID: 22506599

44. Chung The H, Karkey A, Pham Thanh D, Boinett CJ, Cain AK, Ellington M, et al. A high-resolution genomic analysis of multidrug-resistant hospital outbreaks of Klebsiella pneumoniae. EMBO Mol Med. 2015; 7: 227–239. https://doi.org/10.15222/emmm.201404767 PMID: 25712531

45. Onori R, Gaiarsa S, Comandatore F, Pongolini S, Brisse S, Colombo A, et al. Tracking nosocomial Klebsiella pneumoniae infections and outbreaks by whole-genome analysis: Small-scale Italian scenario within a single hospital. J Clin Microbiol. 2015; 53: 2861–2868. https://doi.org/10.1128/JCM.00545-15 PMID: 26135860

46. Hennequin C, Robin F. Correlation between antimicrobial resistance and virulence in Klebsiella pneumoniae. Eur J Clin Microbiol Infect Dis. 2016; 35: 333–341. https://doi.org/10.1007/s10096-015-2559-7 PMID: 26718943

47. Becker L, Kaase M, Pfeifer Y, Fuchs S, Reuss A, von Laer A, et al. Genome-based analysis of carbapenemase-producing Klebsiella pneumoniae isolates from German hospital patients, 2008–2014. Antimicrob Resist Infect Control. 2018; 7: 62. https://doi.org/10.1186/s13756-018-0392-y PMID: 29744043

48. Bachman MA, Oyler JE, Burns SH, Caza M, Lépine F, Dozois CM, et al. Klebsiella pneumoniae yersiniabactin promotes respiratory tract infection through evasion of lipocalin 2. Infect Immun. 2011; 79: 3309–3316. https://doi.org/10.1128/IAI.05114-11 PMID: 21576334

49. Lam MMC, Wyres KL, Judd LM, Wick RR, Jenney A, Brisse S, et al. Tracking key virulence loci encoding aerobactin and salmochelin siderophore synthesis in Klebsiella pneumoniae. Genome Med. 2018; 10: 77. https://doi.org/10.1186/s13073-018-0587-5 PMID: 30371343

50. Wyres KL, Gorrie C, Edwards DJ, Wertheim HF, Hsu LY, Van Kinh N, et al. Extensive capsule locus variation and large-scale genomic recombination within the Klebsiella pneumoniae clonal group 258. Genome Biol Evol. 2015; 7: 1267–1279. https://doi.org/10.1093/gbe/evv062 PMID: 25861820

51. Rojas LJ, Weinstock GM, De La Cadena E, Diaz L, Rios R, Hanson BM, et al. An analysis of the epidemic of Klebsiella pneumoniae carbapenemase-producing K. pneumoniae: convergence of two evolutionary mechanisms creates the “perfect storm”. J Infect Dis. 2018; 217: 82–92.

52. Hancock SJ, Phan M-D, Peters KM, Forde BM, Chong TM, Yin WF, et al. Identification of IncA/C plasmid replication and maintenance genes and development of a plasmid multilocus sequence typing scheme. Antimicrob Agents Chemother. 2017; 61: pii = e01740–16. https://doi.org/10.1128/AAC.01740-16 PMID: 27872077

53. Carattoli A. Plasmids and the spread of resistance. Int J Med Microbiol. 2013; 303: 298–304. https://doi.org/10.1016/j.ijmm.2013.02.001 PMID: 23499304

54. Yamashita A, Sekizuka T, Kuroda M. Characterization of antimicrobial resistance dissemination across plasmid communities classified by network analysis. Pathogens. 2014; 3: 356–376. https://doi.org/10.3390/pathogens3020356 PMID: 25437804

55. Arredondo-Alonso S, Willems RJ, van Schaik W, Schürch AC. On the (im)possibility of reconstructing plasmids from whole-genome short-read sequencing data. Microb Genomics. 2017; 3: e000128.