Multidrug-resistant *Klebsiella pneumoniae*: a retrospective study in Manaus, Brazil

Rafael Nakamura-Silva[1#]. Louise Cerdeira[2,3#]. Mariana Oliveira-Silva[1]. Karen Regina Carim da Costa[4]. Elder Sano[5,6]. Bruna Fuga[5,6,7]. Quézia Moura[8]. Fernanda Esposito[6,7]. Nilton Lincopan[5,6,7]. Kelly Wyres[2]. André Pitondo-Silva[1,9*].

#These authors contributed equally to this work.

[1] Postgraduate Progam in Environmental Technology, Universidade de Ribeirão Preto, Ribeirão Preto, São Paulo, Brazil.

[2] Department of Infectious Diseases, Central Clinical School, Monash University, Melbourne, Australia.

[3] Department of Vector Biology, Liverpool School of Tropical Medicine, Liverpool, UK

[4] School of Pharmaceutical Sciences, Universidade Federal do Amazonas, Amazonas, Brazil.

[5] Institute of Biomedical Sciences, Universidade de São Paulo, São Paulo, Brazil.

[6] One Health Brazilian Resistance Project (OneBR), Brazil.

[7] Department of Clinical Analysis, School of Pharmaceutical Sciences of University of São Paulo, São Paulo, Brazil.

[8] Federal Institute of Education, Science and Technology of Espírito Santo, Vila Velha, Brazil.

[9] Postgraduate Program in Dentistry, Universidade de Ribeirão Preto, Ribeirão Preto, São Paulo, Brazil.

***Address correspondence to**

André Pitondo da Silva, PhD

Universidade de Ribeirão Preto – UNAERP

Bloco J, Laboratório 1. Av. Costábile Romano, 2201 - Ribeirânia,

Ribeirão Preto - SP, Brazil. CEP: 14096-900

Email: andre@pitondo.com.br

Abstract

Klebsiella pneumoniae is an opportunistic pathogen that can cause several infections, mainly in hospitalised or immunocompromised individuals. The spread of *K. pneumoniae* emerging virulent and multidrug-resistant clones is a worldwide concern and its identification is crucial to control these strains especially in hospitals. This article reports data related to multi-resistant *K. pneumoniae* strains, isolated from inpatients in the city of Manaus, Brazil, harbouring virulence and antimicrobial resistance genes, including high-risk international clones belonging to clonal group (CG) 258. Twenty-one strains isolated from different patients admitted to four hospitals in the city of Manaus, located in the state of Amazonas, Northern Brazil (Amazon Rainforest region) were evaluated. The majority of strains (61.9 % n = 13) were classified as multidrug-resistant (MDR), and five strains (23.8 %) as extensively drug-resistant (XDR). Several virulence and antimicrobial resistance genes were found among the strains and eight strains (38.1 %) presented the hypermucoviscous phenotype. MLST analysis demonstrated a great diversity of STs among the strains, totaling 12 different STs (ST11, ST23, ST198, ST277, ST307, ST340, ST378, ST462, ST502, ST3991, ST3993 and ST5209). Three of these (ST11, ST23 and ST340) belong to CG258.

Keywords: *Klebsiella pneumoniae*, resistome, virulome, hypermucoviscous, WGS, CG258.

Introduction

Klebsiella pneumoniae is a member of the *Enterobacteriaceae* family and an opportunistic pathogen that could cause several infections (pneumonia, urinary tract infections, bacteremia, among others), mainly in hospitalised or immunocompromised individuals (Bengoeche et al. 2019).

The appearance and spread of hypervirulent strains have increased the number of people susceptible to infections; also, strains of *K. pneumoniae* have become increasingly resistant to antibiotics, making antibiotic therapy more challenging. Several new antimicrobial resistance genes were discovered in *K. pneumoniae* before spreading to other pathogens; bla_{KPC}, $bla_{\text{OXA-48-like}}$ and $bla_{\text{NDM-1}}$ are examples (Wyres et al. 2018). Molecular epidemiology analyses allow us to determine the global spread of high-risk clones, thus, providing the necessary data to develop strategies to limit the spread of clinically dangerous strains (Mathers et al. 2015).

The purpose of this study was to determine the pathogenic potential and the antimicrobial resistance profiles of *K. pneumoniae* strains isolated from different patients admitted to four hospitals in the city of Manaus, located in the state of Amazonas, Northern Brazil. Besides, to analyse the genetic diversity and epidemiological relationship of the strains. Manaus is a Brazilian municipality, Amazonas capital, and the leading financial centre in the North of the country. It is the most populous city in the entire Brazilian Amazon, located in the centre of the world's largest tropical forest (IBGE, 2019).

Recently, the city suffered a collapse in the public health system due to the COVID-19 pandemic (Ferrante et al. 2020). Therefore, studies even before the pandemic

period aiming to elucidate and prevent the spread of hypervirulent and multidrug-resistant bacteria in hospitals in this region are critical (Ribas et al. 2019).

Material and methods

Bacterial strains

In this study, 21 *K. pneumoniae* strains isolated from different patients admitted to four hospitals in the city of Manaus, located in the state of Amazonas, Northern Brazil (Amazon Rainforest region), were evaluated. The strains were randomly selected from the isolate that emerged from November 2014 to May 2016, from different sources, including urine, blood, tracheal secretion, wound secretion, catheter tip, rectal swab (Figure 1). Bacterial identification was performed by the automated system VITEK 2 (Biomérieux) and confirmed by 16S rRNA sequencing using the primers fD1 (5'-AGAGTTTGATCCTGGCTCAG - 3 ') and rP2 (5'-ACGGCTACCTTGTTACGACTT - 3'), according to Weisburg et al. (1991). Based on the antimicrobial resistance classification, presence of hypermucoviscosity and virulence genes, ten strains were subjected to whole-genome sequencing (WGS): KpAm03, KpAm04, KpAm05, KpAm06, KpAm07, KpAm09, KpAm12, KpAm14, KpAm17 and KpAm21.

Hypermucoviscosity test

For the detection of hypermucoviscous phenotype (HMV), the strains were inoculated on Mueller-Hinton agar (Oxoid), for approximately 18 hours at 37º C. Following bacterial growth, using the bacteriological loop, an isolated colony was touched and raised vertically. The formation of a dense string ≥5 mm was considered a positive HMV phenotype, according to Wiskur et al. (2008).

Antimicrobial susceptibility test

Antimicrobial susceptibility tests were performed by disc diffusion for 40 different antibiotics on Mueller-Hinton agar (Oxoid), according to Clinical Laboratory Standards Institute (CLSI, 2020) recommendations for *Enterobacterales*. Susceptibility to colistin was determined by microdilution, according to CLSI 2020. The antibiotics tested are described in Figure 1. *Escherichia. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as controls in this experiment.

Each strain was considered susceptible or non-susceptible (either intermediate or resistant) to each antibiotic tested. Based on the susceptibility profile, the strains were classified as multidrug-resistant (MDR), extensively drug-resistant (XDR) or pandrug-resistant (PDR), according to Magiorakos et al. (2012).

Detection of virulence and β-lactamase encoding genes

All strains were subjected to conventional Polymerase Chain Reaction *(*PCR) assays to detect 10 virulence genes (*rmpA*, *kfu, allS*, *fimH, mrkD*, *ycfM*, *entB*, *iutA*, K2, *magA*) and 15 β- lactamase encoding genes, including carbapenemases (bla_{GES}, bla_{IMP}, bla_{KPC}, bla_{NDM}, $bla_{OXA\text{-}1\text{-}like}$, $bla_{OXA\text{-}48\text{-}like}$, bla_{VIM}, bla_{SPM} and bla_{GIM}), extended-spectrum β-lactamases - ESBLs ($bla_{CTX\text{-}M\text{-}Gp1}$, $bla_{CTX\text{-}M\text{-}Gp2}$, $bla_{CTX\text{-}M\text{-}Gp9}$, $bla_{CMY\text{-}2}$, bla_{VEB} e bla_{BEL}) and also colistin resistance gene (*mcr*-1).

To confirm the detected genes' identity, an amplicon from each gene was randomly selected for DNA sequencing (ABI 3500xL Genetic Analyzer; Applied Biosystems, Foster City, CA). The obtained sequences were compared with those available in GenBank using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Multilocus Sequence Typing (MLST)

All strains were submitted to the MLST technique using the primers and PCR conditions for protocol 2 in the MLST database for *K. pneumoniae* (http://www.pasteur.fr/recherche/genopole/PF8/mlst/K*pneumoniae*.html).

Whole Genome Sequencing (WGS) and Bioinformatic analysis

Total genomic DNA was extracted using a PureLinkTM Quick Gel Extraction Kit (Life Technologies, CA). Genomic DNA quality and quantity were assessed using a Qubit1 2.0 fluorometer (Life Technologies) and sequenced via 2x150-bp paired-end library on a NextSeq550 or MiSeq platform (Illumina Inc., San Diego, CA), using 250-bp paired-end (PE) reads (Table supplementary 1). Read with a PHRED quality score below 20 were discarded, and adapters were trimmed using TrimGalore v0.6.5 (https://github.com/FelixKrueger/TrimGalore). The reads were *de novo* assembled using Unicycler v0.4.0 (Wick et al. 2017) and annotated using the Prokaryotic Genome Annotation Pipeline v.3.2 (PGAP) NCBI.

Antimicrobial resistance (AMR) genes were identified using ABRicate v1.0.1 (https://github.com/tseemann/abricate) with ResFinder v4.1 database (Bortolaia et al. 2020) and a database for heavy metals and biocides resistance genes. ABRicate was also used with PlasmidFinder v2.1 database (Carattoli et al. 2014) to detect plasmid replicons among the assemblies contigs. MLST, virulence factors, capsule synthesis (K-locus), lipopolysaccharide (O-locus), and mutations on porins and quinolone resistance determining regions were identified using Kleborate v2.0.1 (Lam et al. 2021).

CSI Phylogeny (Kaas et al. 2014) was used to generate an approximately maximum-likelihood phylogenetic tree the assemblies, using *K. pneumoniae* strain HS11286 chromosome (RefSeq accession number NC_016845.1) as reference. The tree

was rooted at midpoint and annotated with data from ABRicate and Kleborate, as well as metadata, using iTOL v6 (Letunic et al. 2021). iTOL was also used with a dummy tree to generate heatmaps with antibiogram and PCR results for all the 21 strains.

Results and discussion

This study evaluated 21 *K. pneumoniae* strains isolated from different patients admitted to four hospitals in Manaus, located in the state of Amazonas, northern Brazil. All strains were subjected to antimicrobial susceptibility test, with a total of forty-one antibiotics of different classes (Figure 1), most strains were non-susceptible to the class folate pathway antagonists, sulphonamide (SUL) (95.2 % n = 20); trimethoprim-sulfamethoxazole (SUT) (85.7 %) and trimethoprim (TRI) (85.7 %); as well nitrofurantoin (NIT) and cefuroxime (CRX) (both 90.5 %); a high rate of non-susceptibility was also demonstrated for β-lactam combination agents, ticarcillin-clavulanate (TAC) and ampicillin-sulbactam (APS) (both 80.9 %). Non-susceptibility rates above 70 % were also observed for the antibiotics: streptomycin (EST), tobramycin (TOB), cefaclor (CFC) and ceftaroline (CTL). The results of non-susceptibility of each antibiotic are shown in Figure 1.

The majority of strains (61.9 % n = 13) were classified as MDR, according to the classification suggested by Magiorakos et al. (2012). Moreover, five strains (23.8 %) were classified as XDR (Figure 1). Ferreira et al. (2019) investigated the resistance profile of 25 *K. pneumoniae* from the Tocantins, northern Brazil, finding a high incidence of MDR among them (84 %). Gonçalves et al. (2017) studied 26 *K. pneumoniae* strains from patients admitted in a tertiary hospital in Londrina, southern Brazil, being 53.8 % classified as MDR and 26.9 % as XDR and 11.5 % PDR. A study conducted by Pereira et al. (2019) evaluated the frequency of different extended-spectrum β-lactamases

(ESBL), associating it with antimicrobial resistance in *E. coli* (362 strains) and *Klebsiella* spp. (73 strains), demonstrate a relevant frequency of ESBL (11 %) and a significantly higher percentage of resistance in 91.3 % (n = 21/23) antimicrobials analysed: ampicillin (AMP), ampicillin-sulbactam (APS), piperacillin-tazobactam (PIT), amoxicillin-clavulanate (AMC), cefazolin (CFZ), cefotaxime (CTX), ceftazidime (CAZ), ceftriaxone (CRO), cefuroxime (CRX), cefepime (CPM), cefoxitin (CFO), aztreonam (ATM), ertapenem (ERT), meropenem (MPM), imipenem (IPM), amikacin (AMI), tobramycin (TOB), nitrofurantoin (NIT), sulfamethoxazole (SUT), tetracycline (TET), ciprofloxacin (CIP), levofloxacin (LEV). The exceptions are APS and IPM. Therefore, these works, corroborate with the results of the present study.

PCR analyses detected in eleven strains (52.4 %) presence of the *bla*$_{\text{OXA-1-}like}$ genes, ten with *bla*$_{\text{CTX-M-Gp1}}$ genes (47.6 %) and seven strains harboring *bla*$_{\text{KPC}}$ (33.3 %). Accession numbers MT330306, MT330308 and MT330310. The XDR KpAm06 and KpAm08 encoded all the three of these β-lactamases genes, another ten strains (KpAm01, KpAm02, KpAm03, KpAm07, KpAm09, KpAm13, KpAm14, KpAm17, KpAm19 and KpAm21) harboured two of these three β-lactamases. The same genes have been reported in *K. pneumoniae* from similar studies in southern, southeastern and northern Brazil (Gonçalves et al. 2017; Azevedo et al. 2019; Ferreira et al. 2019).

Among the virulence genes, the *fimH* gene was detected in majority strains (95.2 % n = 20), the second most prevalent gene was *ycfM* (90.5 % n = 19), followed by *entB* (85.7 % n = 18) and *mrkD* (80.9 % n = 17) (Figure 1). Accession numbers of all detected virulence genes MT330312, MT330313, MT330314, MT330316, MT330317, MT330319, MT330320, MT330322 and MT330324. Remya et al. (2019) studied virulence factors in 370 *K. pneumoniae* clinical strains and showed that *entB* was present in 90 % of the strains, *fimH* 89.1 %, *ybtS* 44.3 %, *kfu* 27.8 %, *rmpA* 5.1 %, K2 2 %, *allS*

1 % and *magA* 0.2 %. The large detection of *entB* and *fimH*; reasonable *ybtS* and; low *rmpA* and *magA* are similar to our results. Fimbrial adhesins (*fimH*, *mrk*D), siderophores (*ent*B, mainly) and lipopolysaccharides (*ycfM*) were the most common genes in this study and similar results were found by Candan et al. (2015), with 74 % *ycfM* and 65 % of *mrkD*, *fimH* and *entB*, which studied 15 *K. pneumoniae* clinical strains isolated from different sources. The high prevalence of *fimH*, *mrkD* and *entB* genes found in this study was already expected, other studies have shown a high correlation of these genes in *K. pneumoniae* strains (Azevedo et al. 2019; Ferreira et al. 2019; Kus et al. 2017). The strain KpAm11 presented nine of the virulence genes studied, only K2 was not found in this strain (Figure 1). Eight strains (38.1 %) presented the HMV phenotype (Figure 1), the *rmpA*2 (mucoid phenotype A2 regulator) and *magA* genes (mucoviscosity-associated A gene) were associated with HMV phenotype, however, subsequent studies showed that the *magA* is associated to specific capsular serotype K1 (*wzy* K1) (Catalán-Nájera et al. 2017). Among the eight strains positive for this phenotypic in our study, only KpAm11and KpAm24 presented *mag*A and *rmp*A genes. Similar results were found by Lee et al. (2010) which found three (8 %) among 35 strains that presented the phenotype without the presence of at least one of the three genes*, magA*, *rmpA* and *rmpA*2. Other studies also showed equivalents results, 10.3 % (n = 6/58) HMV *K. pneumoniae* from Taiwan did not have the *magA* and *rmpA* genes (Yu et al. 2006) and among the results of Mohammed et al. (2018), one out of six clinical strains demonstrated the characteristic in question. Moreover, our findings corroborate the results recently described by our research group (Nakamura-Silva et al. 2021) and also by Garza-Ramos (2015) who did not find these genes in *K. variicola* HMV strains. These results indicate that other genes may be involved in this phenotype in different species of *Klebsiella* which need further investigation, Walker et al. (2020) suggest that the HMV phenotype is probably due to

factors not exclusive to the capsule. These authors suggest that it is necessary to investigate and clarify the link between the HMV phenotype and the capsule.

MLST analysis demonstrated a great diversity of STs among the strains, totaling 12 different STs (ST11, ST23, ST198, ST277, ST307, ST340, ST378, ST462, ST502, ST3991, ST3993 and ST5209). Three of these strains (ST11, ST23 and ST340) belong to the clonal group (CG) 258, which has been globally described as a KPC spreader and is often related to carbapenemase production. Therefore, strains with STs grouped into this CG are classified as international high-risk clones (Munoz-Price et al. 2015; Gonçalves et al. 2017; Azevedo et al. 2019). In fact, among the eight strains harbouring *bla*$_{KPC}$ gene in this study, seven belong to CG258. *K. pneumoniae* strains belonging to CG258 harbouring virulence and resistance genes have been described causing both hospital and community infections (Azevedo et al. 2019). ST11 was found in three strains in the study (KpAm01, KpAm02 and KpAm13), all classified as XDR, this ST is widespread in Brazil and is internationally considered a high-risk clone. ST340 was found in three strains (KpAm06, KpAm08 and KpAm09) with XDR profile in the first two and MDR in the last, and is also known to be widespread in Brazil (Gonçalves et al. 2017). In a study of the genomic population of *K. pneumoniae*, Wyres et al. (2020) describes the six hypervirulent global problem clones, the ST23 found in our strains belong to this group.

The KpAm11 and KpAm24 (ST23), both isolated from tracheal secretion, have the HMV phenotype harboring several virulence genes and *wzi* K1, indicating that these strains are hypervirulent, several other studies describe ST23 worldwide as a hypervirulent clone (Cheng et al. 2015; Mukherjee et al. 2020; Pereira et al. 2017). Moreover, the ST23 was previously described in Brazil in a colistin-resistant *K. pneumoniae* harboring *bla*$_{KPC}$ and several other resistance genes (Boszczowski et al. 2019), Coutinho et al. (2014) reported an invasive liver abscess syndrome caused by *K.*

pneumoniae clone ST23 and also in non-human primates (*Alouatta clamitans*) with hypervirulent and hypermucoid characteristics (Anzai et al. 2017).The ST378 and ST462 are double locus variants of the ST23 and appear in few studies, composing the bacterial collection among several STs (Lin et al. 2016; Zhong et al. 2014; Yan et al. 2015; Zhan et al. 2021; Saxenborn et al. 2021). KpAm16 presented ST307 and Wyres et al. (2019) emphasizes the need for more attentive epidemiological surveillance of this clone, due to its rapid and emerging global spread. This ST was also reported in other studies in southeastern Brazil as described by Dropa et al. (2016), that found *K. pneumoniae* harboring CTX-M-15 from wastewater, and Sartori et al. (2019) related MDR CTX-M-15 causing urinary tract infection in a dog. ST277 is a double locus variant ST258, and was previously reported by Chmelnitsky et al. (2013) in two *K. pneumoniae* ST277 carrying bla_{KPC} gene and the isolate KpAm03 presented the same characteristic. The ST198 and ST502 grouped into smaller CGs, the first one was found in the strains KpAm14, KpAm17 and KpAm21 and was previously described in Brazil in *K. pneumoniae* producing CTX-M-15 isolated from commercial lettuce (Lopes et al. 2017).

The ICE*Kp* is a mobile genetic element associated with invasive infection in *K. pneumoniae* (Lam et al. 2018), where ICE*Kp12* with *ybt* lineage 60 were identified in the WGS analyses in the three ST198 strains. The ST502 was found in three MDR strains (KpAm12, KpAm18 and KpAm19), this ST has been described to cause infections in humans, including community infections in Brazil (Azevedo et al. 2019). Three new STs were determined in this study, ST3991 (KpAm22) due to a new allele number combination, ST3993 (KpAm10), due to the new *inf*B (177), *mdh* (298) and *pho*E (417) alleles and ST5209 (KpAm05), due the new *ton*B (705) allele. Through data analysis by the curators of the MLST database, the KpAm10 strain had its species re-identified as *Klebsiella quasipneumoniae subsp. quasipneumoniae* and was deposited in the database

with this identification. Accession numbers of all WGS strains are listed in Supplementary Table 1.

Conclusion

The results presented in this study showed that more than 80% of the studied strains isolated from inpatients in the city of Manaus, located in the state of Amazonas in Brazil were resistant to several antimicrobials. The majority of strains were classified as MDR and five strains as XDR. Moreover, several virulence and antimicrobial resistance genes were found and eight strains presented the hypermucoviscous phenotype. MLST analysis demonstrated a great diversity among the strains, totaling 12 different STs including high-risk international clones belonging to CG258.

Data

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank, and genomic information of *K. pneumoniae* strains are available on the OneBR platform http://onehealthbr.com/ (Table supplementary 1).

Declarations

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Conflict of interest The authors have declared that no competing interests exist.

Author contributions André Pitondo-Silva conceived and designed the experiments. Rafael Nakamura-Silva conducted all experiments relating to antimicrobial resistance, virulence, MLST and wrote the manuscript. Louise Cerdeira conducted the epidemiological and bioinformatics analyses. Karen R. C. Costa provided the bacterial isolates with their respective information. Mariana Oliveira-Silva supported in the antimicrobial susceptibility experiments. Bruna Fuga and Quézia Moura carried out the genome sequencing. Elder Sano, Fernanda Esposito and Nilton Lincopan performed genomic and bioinformatics analyzes especially in the analysis of resistome, virulome and plasmidome and in the deposit of genomic data. Kelly Wyres contributed especially in the analysis and writing of the manuscript. All authors reviewed and approved the final manuscript.

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Figure legends:

Figure 1: Phenotypic and molecular characterisation of 21 *Klebsiella pneumoniae* isolates from clinical sources in Manaus, Brazil. Antimicrobials tested: amoxicillin-clavulanate (AMC), amikacin (AMI), ampicillin-sulbactam (APS), aztreonam (ATM), ceftazidime (CAZ), cefaclor (CFC), cefixime (CFM), cefoxitin (CFO), cefazolin (CFZ), ciprofloxacin (CIP), chloramphenicol (CLO), colistin (COL), cefepime (CPM), ceftriaxone (CRO), cefuroxime (CRX), ceftaroline (CTL), cefotetan (CTT), cefotaxime (CTX), doripenem (DOR), doxycycline (DOX), ertapenem (ERT), streptomycin (EST), fosfomicin (FOS), gentamicin (GEN), imipenem (IPM), levofloxacin (LEV), lomefloxacin (LMX), minocycline (MIN), meropenem (MPM), nalidixic acid (NAL), netilmicin (NET), nitrofurantoin (NIT), norfloxacin (NOR), ofloxacin (OFX), piperacillin-tazobactam (PIT), sulphonamide (SUL), trimethoprim-sulfamethoxazole (SUT), ticarcillin-clavulanate (TAC), tetracycline (TET), tobramycin (TOB), trimethoprim (TRI).

[a] Colored square: non-susceptible strains; blank squares: susceptible strains. No color was associated to polymyxins, since all strains analysed was susceptible to colistin.

[b] XDR: extensively drug-resistant; MDR: multidrug-resistant; NC: not classified.

[c] Filled circles: detected; unfilled circles: not detected.

Figure 2: Phylogenetic relations, resistome, virulome and plasmidome of 10 *Klebsiella pneumoniae* isolates from clinical sources in Manaus, Brazil.

[a] OneBR ID refers to the ID of the isolates in the One Health Brazilian Resistance Integrated Genomic database (OneBR).

[b] Mutations in quinolone resistance determining regions.

[c] QAC: quaternary ammonium compounds.

Table S1: Whole genome sequencing metadata of 10 *Klebsiella pneumoniae* isolates from clinical sources in Manaus, Brazil.

OneBR ID: refers to the ID of the isolates in the One Health Brazilian Resistance Integrated Genomic database; ND: not deposited.

Figure 1:

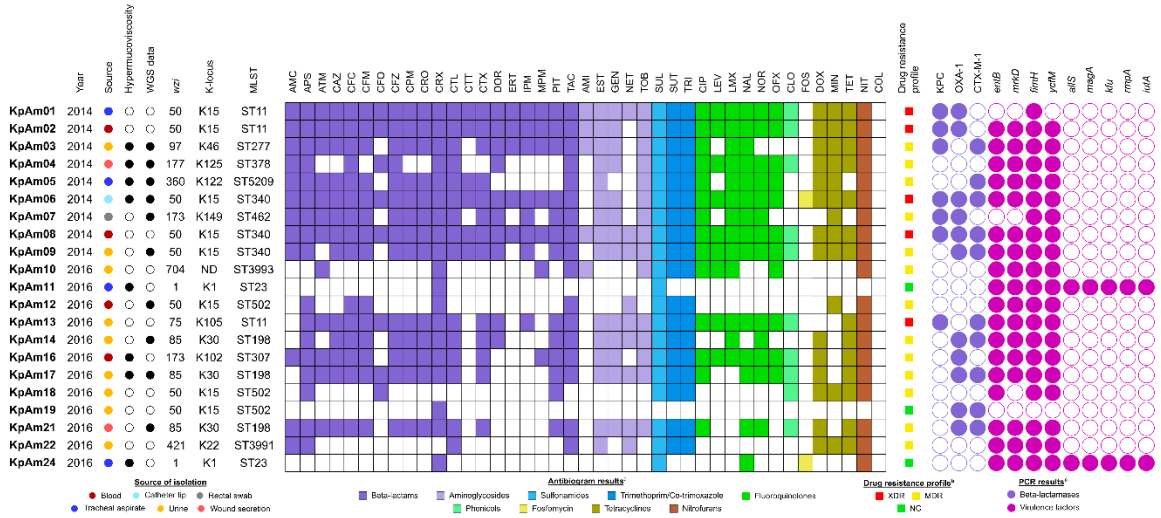

Figure 2:

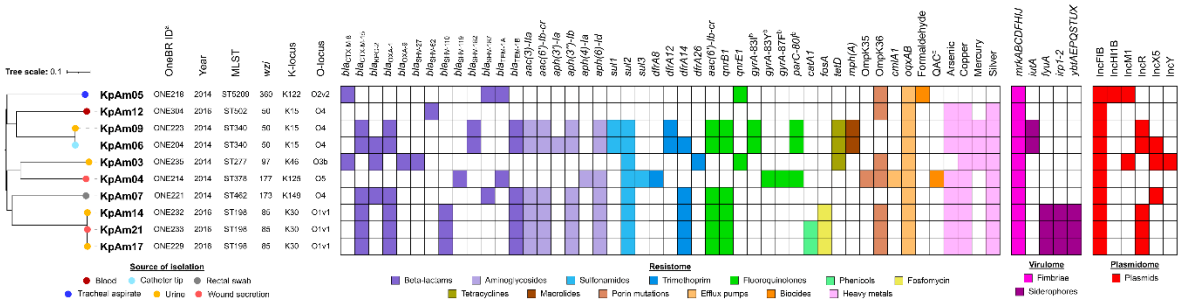

Table S1:

ID	OneBR ID	Isolation source	Year	Illumina platform	Sequencing date	Assembler	Genome (bp)	Contigs no.	Accession number
KpAm03	ONE235	Urine	2014	NextSeq	July, 2019	Unicycler v0.4.8	5672492	206	JAEDZI000000000
KpAm04	ONE214	Wound secretion	2014	NextSeq	July, 2019	Unicycler v0.4.8	5487587	88	JAEDYU000000000
KpAm05	ONE218	Tracheal aspirate	2014	NextSeq	July, 2019	Unicycler v0.4.8	5481240	143	JAEDYW000000000
KpAm06	ONE204	Catheter tip	2014	MiSeq	May, 2017	Unicycler v0.4.8	5481019	117	JAEDYM000000000
KpAm07	ONE221	Rectal swab	2014	NextSeq	July, 2019	Unicycler v0.4.8	5428376	130	JAEDYY000000000
KpAm09	ONE223	Urine	2014	NextSeq	July, 2019	Unicycler v0.4.8	5407560	179	JAEDZA000000000
KpAm12	ND	Blood	2016	NextSeq	July, 2019	Unicycler v0.4.8	5491908	155	JAHVCH000000000
KpAm14	ONE232	Urine	2016	NextSeq	July, 2019	Unicycler v0.4.8	5585714	104	JAEDZG000000000
KpAm17	ONE229	Urine	2016	NextSeq	July, 2019	Unicycler v0.4.8	5612575	247	JAEDZE000000000
KpAm21	ONE233	Wound secretion	2016	NextSeq	July, 2019	Unicycler v0.4.8	5631383	142	JAEDZH000000000