Whole Genome Sequence Analysis of the First Australian OXA-48-Producing Outbreak-Associated Klebsiella pneumoniae Isolates: The Resistome and In Vivo Evolution

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Abstract

Whole genome sequencing was used to characterize the resistome of intensive care unit (ICU) outbreak-associated carbapenem-resistant K. pneumoniae isolates. Importantly, and of particular concern, the carbapenem-hydrolyzing β-lactamase gene blaOXA-48 and the extended-spectrum β-lactamase gene blaCTX-M-14 were identified on a single broad host-range conjugative plasmid. This represents the first report of blaOXA-48 in Australia and highlights the importance of resistance gene surveillance, as such plasmids can silently spread amongst enterobacterial populations and have the potential to drastically limit treatment options. Furthermore, the in vivo evolution of these isolates was also examined after 18 months of intra-abdominal carriage in a patient that transited through the ICU during the outbreak period. Reflecting the clonality of K. pneumoniae, only 11 single nucleotide polymorphisms (SNPs) were accumulated during this time-period and many of these were associated with genes involved in tolerance/resistance to antibiotics, metals or organic solvents, and transcriptional regulation. Collectively, these SNPs are likely to be associated with changes in virulence (at least to some extent) that have refined the in vivo colonization capacity of the original outbreak isolate.

Introduction

Klebsiella pneumoniae is a common cause of infections worldwide, both in community and hospital settings [1,2]. Based on data from the Study for Monitoring Antimicrobial Resistance Trends (SMART), carbapenems remain the most effective treatment option for these infections, especially those caused by strains producing extended-spectrum β-lactamases (ESBLs) [1,2]. While the occurrence of ESBL-producing K. pneumoniae infections has been variable over the past decade, there has been an overall increase in the number of these strains [1,3]. The consequence of ESBL-associated infections is a greater reliance on carbapenems as one of the few remaining effective agents. Therefore, the emergence of carbapenem-resistant K. pneumoniae is particularly worrisome, as not only are treatment options limited but these infections are associated with increased morbidity and mortality [4,5].

In Australia, carbapenem resistance in K. pneumoniae is uncommon and over the past decade has generally been secondary to the expression of metallo-β-lactamase (MBL) genes (specifically blaIMP-4) [6], in combination with changes in outer-membrane porins. Recently, two K. pneumoniae isolates have been reported that produce either the MBL NDM-1 [7] or an Ambler Class A KPC-type carbapenem-hydrolyzing β-lactamase [8]. Furthermore, with respect to Enterobacteriaceae, Ambler class D carbapenem-hydrolyzing β-lactamase (CHDL) genes have also recently emerged in Australia with the report of a clinical K. pneumoniae isolate carrying a plasmid with blaOXA-111 [9]. However, a related gene, blaOXA-48, which was first identified in a K. pneumoniae isolate from Turkey in 2001 [10], and that has spread to Africa, Asia and Europe, has not previously been detected in Australia [11]. The broad dissemination of blaOXA-48, which has largely been due to an association with plasmid-borne Tn1999 or related transposons [11], is of major concern given the ease at which transmission and spread occurs and the subsequent consequence for therapy.

In this study we used whole genome sequencing to characterize the resistome of the first known OXA-48 producing carbapenem-resistant K. pneumoniae isolates following an introduction resulting in an outbreak in a metropolitan Sydney Intensive Care Unit (ICU). In addition, we examine the in vivo evolution of this strain based on recovery of the same isolate from an “outbreak” patient following 18 months of carriage.
Methods

Bacterial Strains, Growth Conditions and Antibiotic Resistance Profiles

Isolates: In 2010, a multi-drug carbapenem-resistant *K. pneumoniae* (Kp001) was introduced into the ICU of a Sydney Metropolitan Hospital by a patient recently returned from Egypt. Three additional patients acquired the organism over several months before termination of the outbreak. All four patients who developed an infection with this organism died. However, 18 months later, a similar *K. pneumoniae* isolate (Kp002) was recovered from the abdominal fluid of a patient (post-hernia repair) who had transited through the ICU at the time of the initial outbreak, despite negative rectal screening swabs at the time of the outbreak. Upon referral to a reference laboratory, both isolates were indistinguishable by either antibiotic resistance profiling or molecular diagnostics (pulsed-field gel electrophoresis and enterobacterial repetitive intergenic consensus sequence PCR; data not shown).

Bacterial strains used in this study are listed in Table 1. Bacterial strains were grown at 37°C in LB medium (Sigma-Aldrich; St. Louis, USA) or on plates containing LB medium and 1.5% w/v agar (Amresco; Solon, USA), unless otherwise stated. When required, media was supplemented with 100 μg mL\(^{-1}\) ampicillin (Amresco; Solon, USA) and/or 100 μg mL\(^{-1}\) rifampicin (Sigma-Aldrich; St. Louis, USA). Antibiotic resistance profiles were determined on a VITEK 2 AST-N149 card using the global and natural resistance database (constructed within CLC Genomics Workbench) of clinically relevant antibiotic resistance genes (see Table S1). BLASTn analyses of resultant contigs using the NCBI non-redundant nucleotide database were also performed in order to examine the genetic context of identified resistance determinants. Gaps in plasmid read mappings were closed via PCR amplification and capillary sequencing.

Conjugation Experiments

Filter-based conjugation experiments were performed as previously described [12] using a rifampicin-resistant mutant *Escherichia coli* DH5α strain (Ec002), which was obtained via growth on an LB agar plate containing 100 μg mL\(^{-1}\) rifampicin.

DNA Manipulations

DNA was extracted from *K. pneumoniae* and *Escherichia coli* cells using the ISOLATE Genomic DNA and Plasmid Mini Kits (Bioline; London, UK), respectively. DNA fragments were PCR-amplified using BioTaq (Bioline; London, UK) and primer pairs described in Table 1. Capillary sequencing of PCR products was performed by Macrogen Inc (Seoul, KOR).

Whole Genome Sequencing

Kp001 DNA was sent to The Ramaciotti Centre (University of New South Wales; Sydney, AUS) for sequencing on an Illumina HiSeq 2000 system (Illumina Inc; San Diego, USA). A fragment library of Kp002 DNA was generated and sequenced on an Ion Torrent PGM (Life Technologies; Carlsbad, USA) according to the manufacturer’s instructions. Analysis of Kp001 and Kp002 genomic data was performed using CLC Genomics Workbench 5.5 (CLC bio; Katrinebjerg, DEN). Reference mapping of reads to the genome of the non-multi-drug resistant *K. pneumoniae* strain NTUH-K2044 (GenBank accession no. AP006725) facilitated variant analysis using a quality-based algorithm (as implemented in CLC Genomics Workbench) applying an 80% genotype frequency cutoff with a minimum coverage of 10 reads. Homopolymer variants as well as variants present in both Kp001 and Kp002 were excluded. The remaining variants were curated manually to ensure accurate identification. Raw data for this project has been uploaded to the Sequencing Read Archive under accession number SRA062913. A *de novo* assembly of reads that did not map to NTUH-K2044 was used to query an in-house database generating a fragment library (constructed within CLC Genomics Workbench) of clinically relevant antibiotic resistance genes (see Table S1). BLASTn analyses of resultant contigs using the NCBI non-redundant nucleotide database were also performed in order to examine the genetic context of identified resistance determinants. Gaps in plasmid read mappings were closed via PCR amplification and capillary sequencing.

Table 1. Bacterial strains, plasmids and primers.

| Strain, plasmid or primer | Genotype, relevant characteristics or sequence | Source |
|---------------------------|-----------------------------------------------|--------|
| **Strains E. coli**        |                                               |        |
| Ec002                     | Rifampicin® derivative of DH5α: F- endA hsdR17 supE44 thi-1 λ- recA1 gyrA96 relA1 d180 d LacZAM15 | This study |
| Ec003                     | Ec002 carrying pJEG011 and pJEG012            | This study |
| *K. pneumoniae* Kp001     | Clinical outbreak-associated isolate carrying pJEG011 and pJEG012 | This study |
| Kp002                     | Clinical outbreak-associated isolate phenotypically and genotypically indistinguishable from Kp001: isolated after 18 months of intra-abdominal carriage | This study |
| **Plasmids**              |                                               |        |
| pJEG011                   | IncL/M, Tn1999, ISEcpl1-bla<sub>CTX-M-14</sub>, Tn538J/A | This study |
| pJEG012                   | pir, Tn1337                                    | This study |
| **Primers**               |                                               |        |
| aacA4-F                   | 5'- ggagagttgatggattctacctc -3'                | This study |
| aacA4-R                   | 5'- ggaggggttcaacac -3'                       | This study |
| aacC2-F                   | 5'- gttgtagagagatctgccagt-3'                  | This study |
| aacC2-R                   | 5'- ttgctgacggycttccta -3'                    | This study |
| CTX-M-14-F                | 5'- gaagagctgaagagagatg -3'                   | This study |
| CTX-M-14-R                | 5'- tcgctgctttctttatg -3'                     | This study |
| OXA-48-F                  | 5'- ggttccacctataattgag -3'                   | This study |
| OXA-48-R                  | 5'- cgagcatcaagcatatttgc -3'                  | This study |

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**Results and Discussion**

**Defining the Common Resistome**

The **mobile resistome**. Both Kp001 and Kp002 were shown to be resistant to aminoglycosides and most β-lactams including, and of particular concern, meropenem. In order to fully define the resistome for both isolates, WGS reads that did not map to NTUH-K2044 were **de novo** assembled and examined via BLASTn analysis using an in-house database (created within CLC Genomics Workbench 5.5) of known antibiotic resistance determinants in Gram-negative bacteria (see Methods). Included in the local resistance gene database (RGD) were representative alleles of common aminoglycoside resistance genes [13,14], quinolone resistance genes [15] and β-lactam resistance genes, including those that encode extended-spectrum and carbapenem-hydrolyzing β-lactamases [16–20]. Further BLASTn analysis and reference mapping was then performed in order to determine the genetic context of the identified determinants.

Based on interrogation of the RGD, four β-lactamase genes were identified in both Kp001 and Kp002: **bla**<sub>SHV-1</sub> (which is ubiquitous in *K. pneumoniae*), **bla**<sub>CTX-M-14</sub> (which differs from **bla**<sub>CTX-M-9</sub> by 4 nucleotides), **bla**<sub>OXA-9</sub> and, of particular importance, the **bla**<sub>OXA-48</sub> CHDL gene. Further analysis revealed **bla**<sub>CTX-M-14</sub> was present as part of an IS<sub>Ecp1</sub> transposition unit which had inserted into a plasmid designated pJEG011 (GenBank accession no. KC354801). pJEG011 shares 95% nucleotide sequence similarity with the backbone structure of the multi-resistance IncL/M conjugative plasmid pOXA-48a (GenBank accession no. JN626286) (11), including Tn<sub>1999</sub> containing the **bla**<sub>OXA-48</sub> gene [21] (Figure 1A).

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**Figure 1. Structural features of plasmids pJEG011 and pJEG012.** Comparisons to related plasmids pOXA-48a (A) and pJE143 (B) are shown, respectively; plasmid backbones are represented by thick gray lines and areas of ≥99% sequence identity between plasmids are indicated by the light blue area. Only the following selected genes are annotated and represented by colored arrows: plasmid replication genes, red; transposon-related genes, orange; plasmid partitioning, maintenance (e.g., toxin/antitoxin systems (T/AT)), mobilization and conjugation genes, yellow; aminoglycoside resistance genes, green; β-lactam resistance genes, blue. Dashed arrows represent more than one gene or open reading frame. Insertion sequences (IS) are represented by orange pentagons with the IS number indicated within; the direction of the IS with respect to the transposase gene is indicated by the point of the pentagon. Inverted repeats associated with IS and transposons are indicated by vertical orange lines; the nucleotide sequences of the direct repeats resulting from IS and transposon insertion are indicated above or below the plasmid figures. Integron gene cassettes are represented by orange rectangles.

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Table 2. β-lactam MICs and PCR results for Kp001 and Ec003.

| Antibiotic* | MICs (mg/L)b | Kp001 | Ec003 (Ec002 transconjugant) | Ec002 |
|-------------|-------------|-------|-----------------------------|-------|
| Amoxicillin | ≥32         | ≥32   | ≤2                          |       |
| Amoxicillin/CLA | ≥32     | 16    | ≤2                          |       |
| Ticarcillin/CLA | ≥128     | ≥128  | ≤8                          |       |
| Piperacillin/TZB | ≥128    | ≥128  | ≤4                          |       |
| Cefazolin   | ≥64         | ≥64   | ≤4                          |       |
| Cefoxitin   | ≥64         | ≤4    | ≤4                          |       |
| Ceftazidime | 4           | ≤1    | ≤1                          |       |
| Ceftriaxone | ≥64         | 32    | ≤1                          |       |
| Cefepime    | ≥64         | ≤1    | ≤1                          |       |
| Meropenem   | ≥16         | ≤0.25 | ≤0.25                       |       |
| Gene Target | aacA4       | +     | –                           |       |
|             | bIaTX-M.14  | +     | –                           |       |
|             | bIaOXA-48   | +     | –                           |       |
|             | aacC2       | +     | –                           |       |

*Antibiotic abbreviations: CLA, clavulanic acid, TZB, tazobactam.

Table 3. Antibiotic MICs for Kp001 and Kp002.

| Antibiotic* | MICs (mg/L)b | Kp001 | Kp002 |
|-------------|-------------|-------|-------|
| Amoxicillin | ≥32         | ≥32   | ≥32   |
| Amoxicillin/CLA | ≥32     | ≥32   | ≥32   |
| Ticarcillin/CLA | ≥128     | ≥128  | ≥128  |
| Piperacillin/TZB | ≥128    | ≥128  | ≥128  |
| Cefazolin   | ≥64         | ≥64   | ≥64   |
| Cefoxitin   | ≥64         | ≥64   | ≥64   |
| Ceftazidime | 4           | 4     | 4     |
| Ceftriaxone | ≥64         | ≥64   | ≥64   |
| Cefepime    | ≥64         | ≥64   | ≥64   |
| Meropenem   | ≥16         | ≥16   | ≥16   |
| Amikacin    | ≥64         | ≥64   | ≥64   |
| Gentamicin  | ≥16         | ≥16   | ≥16   |
| Tobramycin  | ≥16         | ≥16   | ≥16   |
| Nalidixic acid | ≥32      | ≥32   | ≥32   |
| Ciprofloxacin | ≥4        | ≥4    | ≥4    |
| Norfloxacin | ≥16         | ≥16   | ≥16   |
| Trimethoprim | 1          | 1     | 1     |
| TMP/SXT     | ≤20         | ≤20   |       |

*Antibiotic abbreviations: CLA, clavulanic acid, TZB, tazobactam; TMP/SXT, trimethoprim/sulfamethoxazole.

The aminoglycoside resistances genes aphaA6 and strAB were also present as part of pJE011, and were located within a novel Tn5393 module (Figure 1A). The other aminoglycoside resistance genes detected (aacC2, aacA4 and adaA1) were not part of pJE011. Further analysis revealed that the aacC2 gene was located on a contig that is flanked by two copies of IS26 in the same orientation as previously described for certain IncFII plasmids [22], however the genetic context of this determinant remained unclear. In contrast, both the aacA4 and adaA1 genes were located on the same contig along with blaOXA-16, and these were all present as part of Tn1331. Further examination of this contig revealed that Tn1331 had inserted into a par-type plasmid designated pJE012 (GenBank accession no. KC534802), which shares >85% nucleotide sequence similarity with the backbone structure of another plasmid, pJE143 (GenBank accession no. JN194214) [23]; pJE012 also contained a putative toxin/anti-toxin system and a single copy of IS26 (Figure 1B).

Contribution of single nucleotide variants. Both isolates had SNPs in gyrA and parC, which encode subunits of DNA gyrase and topoisomerase IV, respectively, and are involved in DNA replication and segregation. These SNPs result in amino acid changes within the quinolone resistance determining regions of GyrA (S80I) and ParC (S83Y and D87G), and collectively are known to confer high-level resistance to ciprofloxacin and nalidixic acid [24].

Transfer of the Mobile Resistome

Conjugation experiments revealed that pJE011 and pJE012 could be readily transferred from Kp001 to Ec002. The presence of both plasmids in a single E. coli transconjugant, Ec003, was determined by PCR amplification of blaCTX-M-14, blaOXA-48 and aacA4 (Table 2). The aacC2 gene was not detected by PCR in the transconjugant (in agreement with the above analysis), suggesting that it is most likely located on the chromosome. Despite increased β-lactam MICs, Ec003 was fully susceptible to meropenem.
**Table 4. SNPs present in Kp002.**

| Mutation | Gene or Locus | Function | Amino Acid Change |
|----------|---------------|----------|------------------|
| C→A (96,127) | KP1_0101 | putative LysR-type transcriptional regulator | T131N |
| A→G (228,794) | gapB | RNA polymerase, β subunit | D527G |
| A→T (676,116) | cusS | copper-sensing two-component system sensor kinase | S446C |
| C→T (1,767,550) | yIC | ABC transport system periplasmic binding component | P258S |
| A→C (2,910,808) | slyA | Transcriptional regulator | V120G |
| C→T (2,960,076) | Upstream of KP1_3109 | putative LysR-type transcriptional regulator | – |
| G→A (3,490,471) | gyrF | DNA gyrase inhibitor | A99V |
| C→A (3,721,779) | glpC | sn-glycerol-3-phosphate dehydrogenase K, small subunit | P383T |
| A→G (4,360,381) | Intergenic | – | – |
| G→A (4,380,149) | Intergenic | – | – |
| G→A (5,084,490) | KP1_5543 | putative acetyltransferase | G120Stop |

*Nucleotide change (genetic location in K. pneumoniae strain NTUH-K2044).*

*Location as annotated in K. pneumoniae strain NTUH-K2044.*

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**Concluding Remarks**

To the best of our knowledge, this study represents the first report of the *blaOXA-48* CHDL gene in Australia. This study also illustrates the in vivo evolution of a multidrug-resistant *K. pneumoniae* isolate during 18 months of carriage. Of note, some of the SNPs identified, particularly those associated with transcriptional regulators, may be involved in modulation of Kp002 virulence capacity. In a global context, this is also the first report of *blcCTX-M-11* and *blaOXA-48* co-residing on a single broad host-range conjugative plasmid (*i.e.*, pJEG011). While international travel has facilitated the clonal spread of *blaOXA-48*-containing *K. pneumoniae* ST101 isolates [36–38], especially from countries along the Mediterranean Sea, the presence of *blaOXA-48* within *Tn1999* (and related transposons) on different Inc group plasmids [21,39,40], has played a crucial role in its dissemination. The emergence of plasmids such as pJEG011, and the one recently described by Potron et al. [41], is of great clinical concern as they have the potential to more broadly disseminate resistance associated with these determinants. In this respect, it is also concerning that these determinants have the potential to go undetected based on antibiotic susceptibility profiles. Therefore, this study highlights the importance of surveillance based on resistance screening, especially in environments where antibiotic selection pressure is prevalent.

**Supporting Information**

Table S1 List of antibiotic resistance genes used in the in-house database for resistome determination. (XLSX)

**Author Contributions**

Conceived and designed the experiments: BAE MAC IBG SJvH SOJ. Performed the experiments: BAE JAS HZ. Analyzed the data: BAE JAS SOJ. Contributed reagents/materials/analysis tools: SMG MAC IBG SOJ. Wrote the paper: BAE SJvH SOJ.

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