Genomic investigation of an outbreak of carbapenemase-producing *Enterobacter cloacae:* long-read sequencing reveals the context of *bla*IMP4 on a widely distributed IncHI2 plasmid

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40-word summary: Whole genome sequencing of *bla*$_{IMP-4}$-producing *Enterobacter cloacae* detected an unknown persistent source of infection within the hospital. All isolates were found to carry multiple antibiotic resistance genes, located in a large multidrug resistant region on a 330,060 bp IncHI2 plasmid.

Key words: *bla*$_{IMP-4}$; carbapenem resistant, whole genome sequencing; plasmid; carbapenemase-producing Enterobacteriaceae (CPE)

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Abstract

Background: We describe whole genome sequencing (WGS) to analyse a cluster of *bla*$_{\text{IMP-4}}$ carbapenemase-producing *Enterobacter cloacae*.

Methods: A cluster of carbapenemase-producing *E. cloacae* were identified over a two month period in 2015 within an Intensive Care Unit (ICU)/Burns Unit in Brisbane, Australia. Phylogenetic relationships based on core single nucleotide polymorphisms (SNPs) were determined using WGS. Genomic comparisons were made to IMP-producing Enterobacteriaceae from neighbouring hospitals and to publicly available genomes to contextualise the isolates in the broader community. Pacific Biosciences Single Molecule Real-Time (SMRT) sequencing of one IMP-4-producing *E. cloacae* strain was used to resolve the full context of the resistance genes.

Results: All outbreak strains were sequence type 90 and differed by only four core SNPs. WGS analysis unequivocally linked all 10 isolates to a 2013 isolate from the same ward, confirming the hospital environment as the most likely original source of infection in the 2015 cases. No clonal relationship was found to IMP-4-producing isolates identified from other local hospitals. However, all IMP-4-producing strains were found to possess an identical *bla*$_{\text{IMP-4}}$ carried on a large IncHI2 plasmid.

Conclusions: During the course of an outbreak investigation, WGS revealed the transmission dynamics of a carbapenemase-producing *E. cloacae* cluster, linking it to a historical isolate from the same Unit and revealing the full context of *bla*$_{\text{IMP-4}}$ on a multi-drug resistant IncHI2 plasmid that appears to be widely distributed in Australia.

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Introduction:

Carbapenem antibiotics have become the mainstay of therapy for serious infections caused by multidrug resistant (MDR) Gram-negative bacteria, especially for strains expressing extended-spectrum beta-lactamase (ESBL) or AmpC-type enzymes [1]. Increased use has driven resistance to carbapenems and the emergence of carbapenemase-producing Enterobacteriaceae (CPE) and carbapenem-resistant Enterobacteriaceae (CRE), which include common enteric species such as *Escherichia coli*, *Klebsiella pneumoniae* and *Enterobacter* spp. [2].

Before 2005, an estimated 99.9% of Enterobacteriaceae were susceptible to carbapenems [3]. However, the isolation of CRE has since increased dramatically and these organisms are now reported in all WHO health regions [4]. The mortality rates for CRE infections are reported to be as high as 48% [5], and resistance to last-line antibiotics used in lieu of carbapenems, such as colistin, has also emerged [6].

Resistance to carbapenems in Enterobacteriaceae occurs via a range of mechanisms. Of greatest concern is the acquisition of genes encoding carbapenemases [7]. This most frequently occurs via transfer of mobile genetic elements, such as plasmids, occasionally carrying multiple β-lactamases co-located with other resistance determinants, rendering these strains MDR or extensively drug-resistant (XDR) [8]. Australia has experienced low rates of CRE [9], although sporadic introduction of *K. pneumoniae* carbapenemase (KPC) [10] and New Delhi metallo-beta-lactamase (NDM) [11] has been reported, including significant nosocomial outbreaks [12]. The most frequently encountered carbapenemase in Australia is $bla_{\text{IMP-4}}$, particularly in *Enterobacter* spp. [13]. IMP-producing *Enterobacter* spp. have caused occasional outbreaks within intensive care or burns units in Australian hospitals [14-16].

Here, we describe the use of whole genome sequencing (WGS) to investigate an outbreak of IMP-4-producing *Enterobacter cloacae* within an Intensive Care Unit (ICU) and Burns facility.

Clinical case report

Two patients in mid 2015 were transferred from regional Queensland hospitals to the ICU with burn injuries sustained from the same accident (Figure 1). *E. cloacae* was cultured from the endotracheal tube (ETT) of patients 1 and 2 on day 6 and 8 of admission, respectively. Both *E. cloacae* were confirmed as MDR by phenotypic testing used in the diagnostic setting (Table 1). Real-time PCR amplification of $bla_{\text{IMP-4}}$ confirmed their status as carbapenemase-producers. Both of these patients were previously well, with no prior hospital admission or contact with healthcare facilities. Neither had been resident or hospitalized overseas for more than 20 years.

Patient 1 underwent debridement and split skin grafting for 29% total body surface area burns on day 2 of ICU admission and subsequently had 3 procedures in the burns operating rooms (Figure 1). An additional MDR-*E. cloacae* was isolated from urine on day 21, eight days after discharge from the ICU. After no further colonisation of MDR-*E. cloacae,* Patient 1 was discharged from the hospital on day 38.

Patient 2 underwent multiple grafting and debridement procedures and was discharged from the ICU on day 17 (Figure 1). MDR-*E. cloacae* colonisation from the ETT and from urine was noted on day 8 and day 15, respectively. By day 19, the patient developed clinical signs of sepsis, with a phenotypically identical isolate identified in blood cultures and from a central venous line (CVL) tip culture. She received piperacillin/tazobactam 4.5 grams 8-hourly for 2 days, improved following line removal and did not receive further antibiotics for this episode. A subsequent *E. cloacae* isolated from urine collected from a urinary catheter 17 days later demonstrated a different

antibiogram with susceptibility to third generation cephalosporins, meropenem and gentamicin. She received 3 days of oral norfloxacin 400mg twice daily with microbiological resolution.

Patient 3, a 39-year old woman, was admitted with 66% total body surface area burns to the same ICU 5 weeks after Patient 1 and 2 were admitted and 20 days after they had been discharged from the ICU (Figure 1). MDR-*E. cloacae* was cultured from the ETT of Patient 3 on day 12 of ICU admission. She had frequent brief admissions to several hospitals since 2010 (never to ICU), and no MDR Gram-negative bacilli were identified in clinical or screening samples during previous admissions. MDR-*E. cloacae* with *Pseudomonas aeruginosa* were isolated from 8 skin swabs and an additional ETT aspirate. On days 19 and day 21, MDR-*E. cloacae* were isolated from blood cultures in the context of skin graft breakdown and signs of systemic inflammatory response syndrome (SIRS) with increasing inotrope requirements (Figure 1). *Streptococcus mitis* was cultured from blood on day 19. On day 36, her condition worsened with signs of SIRS. Transesophageal echocardiography demonstrated aortic and mitral valve lesions consistent with endocarditis. Pancytopenia developed, with a bone marrow aspirate and trephine suggestive of peripheral consumption. Multiple suspected cerebral, pulmonary, splenic and renal septic emboli were identified on imaging. She was palliated on day 47 of admission due to extensive cerebral emboli (Figure 1).

Materials & Methods

Study setting

Primary isolates were obtained from patients admitted to the Royal Brisbane & Women's Hospital (RBWH), a tertiary referral hospital with 929 beds in South-East Queensland, Australia. Additional IMP-producing isolates, cultured from patients admitted to other hospitals in the metropolitan Brisbane area (referred to as Hospital A and B), were obtained from the Central Laboratory of Pathology Queensland for comparison (Table S3).

148

149 **Antimicrobial susceptibility testing and carbapenemase detection**

150 All bacterial isolates were identified by matrix-assisted laser desorption/ionization mass
151 spectrometry (MALDI-TOF) (Vitek MS; bioMérieux, France). Antimicrobial susceptibility testing
152 was carried out using Vitek 2 automated AST-N426 card (bioMérieux) with Etest to determine
153 MICs for meropenem, imipenem and ertapenem. Carbapenemase activity was assessed by the use
154 of the Carba-NP test (RAPIDEC; bioMérieux) and the presence of the $bla_{\text{IMP-4-like}}$ carbapenemase
155 gene confirmed using an in-house multiplex real-time PCR (also targeting NDM, KPC, VIM and
156 OXA-48-like carbapenemases) [17].

157

158 **Bacterial DNA extraction**

159 Single colonies were selected from primary bacterial cultures and grown in 10 mL Lysogeny broth
160 (LB) at 37°C overnight (shaking 250rpm). DNA was extracted using the UltraClean® Microbial
161 DNA Isolation Kit (MO BIO Laboratories) as per manufacturer instructions.

162

163 **Genome sequencing, Quality Control and De Novo Assembly**

164 All isolates in this study were sequenced using Illumina (see supplementary appendix). Reads
165 passing quality control (QC) were assembled using Spades v3.6.0 [18] under default parameters
166 (without careful flag). Contigs with coverage less than 10x were removed from final assemblies.
167 Final assembly metrics were checked using QUAST v2.3 [19] (Table S3).

168

169 **Phylogenetic analysis**

170 SHRiMP (v2.2.3) [20] (as implemented in Nesoni v0.130 [21] under default settings) was used to
171 determine core single nucleotide polymorphisms (SNPs) between the ten 2015 RBWH *E. cloacae*
172 genomes to the reference Ecl1 and create a minimal-spanning tree. Further details of the Ecl1
173 assembly and SNP-calling process are provided in the supplementary appendix. Maximum likelihood

trees of Ecl1 and the 6 *E. cloacae* from hospitals A and B were built using RAxML (v8.1.15) [22] based on the Nesoni core SNPs. RAxML was run with the GTRGAMMA nucleotide substitution rate and an initial seed length of 456 (bootstrap 1000 with Lewis ascertainment correction). Core genome size was estimated using Parsnp v1.2 [23].

Multi-locus Sequence Typing (MLST), Plasmid Typing and Antimicrobial Resistance (AMR) Gene Profiling

MLST of isolate raw reads was performed using srst2 v0.1.5 [24] with typing schemes available on PubMLST (http://pubmlst.org/). Plasmid replicon typing was done based on Compain *et al.* [25]. Antibiotic resistance genes were detected using the ResFinder database [26] and the ARG-ANNOT database [27] with BLASTn and srst2 [24] respectively. Manual confirmation was carried out using BLASTn and read mapping using Burrows-Wheeler Aligner (BWA v0.7.5a-r405) [28]. Further details of whole genome comparisons and phage analysis are given in the supplementary appendix.

Pacific Biosciences (PacBio) Single Molecule Real-Time (SMRT) Sequencing

A representative *E. cloacae* isolate from patient 1 (MS7884) was grown on LB agar at 37°C overnight. IMP positive colonies (determined by colony PCR) were grown overnight in 15 ml LB broth with 2 µg/ml meropenem to avoid plasmid loss. Genomic DNA was extracted using UltraClean® Microbial DNA Isolation Kit (MoBio) as per manufacturer instructions. 18.7 µg of DNA was prepared for sequencing using an 8-12 kb insert library and sequenced on a PacBio RSII sequencer using 1 SMRT cell. Further details of the assembly, annotation methods and plasmid stability in MS7884 are given in the supplementary appendix.

Accession numbers

Genome data has been deposited under Bioproject PRJNA383436. Illumina raw reads (SRS2350257-SRS2350273) and PacBio raw reads (SRX2999346-SRX2999347) have been

deposited in the Sequence Read Archive. The MS7884A chromosome (CP022532), pMS7884A plasmid (CP022533), and pMS7884B plasmid (CP022534) have been deposited in GenBank.

Results

All three patients carry carbapenemase-producing *E. cloacae*

With the exception of MS7889 (isolated from the urine of Patient 2 on day 36), all *E. cloacae* isolates collected from the outbreak were resistant to ceftriaxone, ceftazidime, ticarcillin-clavulanate, piperacillin-tazobactam, meropenem, gentamicin and trimethoprim-sulphamethoxazole by Vitek 2 testing (Table 1) and demonstrated carbapenemase production by Carba-NP. The MICs for meropenem were considerably lower when tested by Etest [29], often falling below the clinically susceptible breakpoint defined by EUCAST, but above the epidemiological cut-off (ECOFF) [30]. MS7889 was fully susceptible to carbapenems (meropenem MIC=0.032 by Etest) and was negative for IMP-4-like genes by PCR (Table 1).

Whole genome sequencing identifies a link to a previous IMP-producing isolate

WGS of 10 isolates from patients 1, 2 and 3 was initiated after microbiological confirmation of a $bla_{\text{IMP-4}}$ *E. cloacae* isolate from a third patient from the RBWH ICU (Figure 1). *In silico* MLST showed all belonged to sequence type (ST) 90 with the majority exhibiting the same resistance gene profile, including a 100% identical $bla_{\text{IMP-4}}$ gene (Table 1). The exception was the carbapenem susceptible isolate MS7889, which was confirmed by WGS to have lost the $bla_{\text{IMP-4}}$ gene as well as several additional resistance genes conserved in the other *E. cloacae* isolates (Table 1). All ten isolates contained an IncHI2 plasmid. Sequence analysis suggests that AmpC derepression is unlikely to contribute to carpabenemase activity in these strains (further details are given in the supplementary appendix).

Comparison of the *E. cloacae* genomes to publicly available draft assemblies identified a close match to *E. cloacae* Ecl1 (GenBank: JRFQ01000000), an ST90 strain isolated from a burns patient at the RBWH ICU almost two years prior to the 2015 outbreak [13, 31]. Antibiotic resistance profiling of the Ecl1 genome revealed an identical resistance profile compared to the majority of the 2015 isolates (Table 1).

The 2015 outbreak isolates were near identical at the core genome level to an isolate from 2013

To investigate the relationship between the isolates at single-nucleotide resolution, reads from the 2015 RBWH isolates were mapped to *E. cloacae* draft assembly for Ecl1. All 2015 RBWH isolates differed by fewer than five core SNPs (4,934,357 bp core genome), consistent with a direct ancestral relationship (Figure 2). Two isolates from Patient 1 and two isolates from Patient 3 were indistinguishable at the core genome level (Figure 2), although all of the isolates from Patient 3 had lost a prophage region (refer supplementary appendix). Ecl1 (isolated in 2013) was very closely related to these isolates, differing by only one core SNP. All four isolates from Patient 2 contained a discriminatory single-nucleotide deletion, thereby ruling out Patient 2 to Patient 3 transmission (Figure 2).

Integration of WGS with infection control response

WGS analysis unequivocally linked all 10 isolates to the 2013 isolate Ecl1 from the same ward, confirming that the clone had not been an incursion from the accident affecting Patient 1 and 2 and that the hospital environment was suspected as the most likely original source of infection in the 2015 cases. In response, 28 environmental samples from the ICU, burns wards and operating theatres were collected 65 days after patient 1 and 2 were admitted and inoculated onto MacConkey agar with 8 mg/mL gentamicin (laboratory standard screening medium for MDR Gram-negative bacilli). No carbapenemase-producing *Enterobacter* spp. were detected. Additionally, no

carbapenemase-producing *Enterobacter* spp. were detected in patients admitted to the ICU or burns unit for a 6-month period following the outbreak.

Sequencing of additional CPE isolates identify a circulating IMP-4-carrying plasmid in Queensland

To determine the broader context of IMP-producing Enterobacteriaceae in surrounding hospitals, seven additional $bla_{\text{IMP-4}}$ producing Enterobacteriaceae (*E. cloacae* n=6, *E. coli* n=1) were sequenced. These represented all $bla_{\text{IMP-4}}$ producing Enterobacteriaceae identified from Brisbane public hospitals via Pathology Queensland for 2015. Both MLST and SNP analysis found no relationship to the 2015 RBWH *E. cloacae*, with approximately 50,000 SNP differences between the ST90 representative strain Ecl1 and its nearest non-ST90 phylogenetic neighbour (Figure 3, also see supplementary appendix). Despite not being clonally related, all additional Enterobacteriaceae isolates possessed very similar antibiotic resistance gene profiles (Table S3), suggesting the possibility of lateral gene transfer via mobile genetic elements (e.g. integrons and/or plasmids). WGS analysis revealed that all 18 CPE isolates in this study, including the *E. coli* isolate, harbored an IncHI2 plasmid (plasmid ST1) and an identical $bla_{\text{IMP-4}}$ gene, strongly suggesting plasmid-mediated circulation of $bla_{\text{IMP-4}}$ between Enterobacteriaceae in Brisbane hospitals.

$bla_{\text{IMP-4}}$ resides in the class 1 integron In809 on an IncHI2 plasmid

Due to the presence of multiple repetitive elements surrounding $bla_{\text{IMP-4}}$, including insertion sequences (IS) and two suspected integrons with similar gene content, we were unable to accurately resolve the context of $bla_{\text{IMP-4}}$ using Illumina sequencing alone. One representative isolate (MS7884) was sequenced twice using PacBio SMRT sequencing, which was able to resolve a complete closed chromosome of 4,810,853 bp and two plasmids: pMS7884A, a 330,060 bp IncHI2 plasmid carrying $bla_{\text{IMP-4}}$ within a ~55 kb MDR region (Figure 4A), and pMS7884B, a smaller untypeable plasmid of 126,208 bp. The pMS7884A MDR region harbours two different class 1

integrons (In37 and In809) as well as a composite transposon conferring resistance to tetracycline and chloramphenicol (Figure 4A). BLASTn and read-mapping analysis revealed the presence of identical plasmids in all but one of the 18 isolates sequenced by Illumina in this study: isolate MS7889 is predicted to have lost a ~34 kb region from its MDR plasmid, including $bla_{\text{IMP-4}}$, due to homologous recombination between two almost identical aminoglycoside resistance genes (Figure 4B). Notably in 15% of cases, sub-culture of MS7884 in the absence of meropenem selection resulted in loss of $bla_{\text{IMP-4}}$ or the entire plasmid. Further details of the complete MS7884 genome and plasmid analysis are presented in the supplementary appendix.

Discussion

While there has been a dramatic improvement in the cost and availability of whole genome sequencing (WGS), it is not clear how these advances can best be incorporated into routine clinical microbiology. Several studies have demonstrated the ability of WGS to provide optimal discrimination between strains to help inform a response to outbreaks or nosocomial acquisition [32-35]. Here, we demonstrate that WGS can help rapidly characterize an outbreak in a critical care setting, particularly regarding transmission pathways.

The finding that the outbreak strains were virtually indistinguishable from an IMP-4-producing *E. cloacae* isolated two years previously from the same unit was unexpected and highlighted the need to consider environmental sources and potential person-to-person transmission, as has been previously described in Australian ICU and burns units [14]. Although we were unable to isolate any IMP-producing *Enterobacter* spp. from environmental sampling, it is possible that this may have been due to enhanced cleaning and additional infection control measures. Healthcare workers are also a possible reservoir, with previous studies confirming carriage of a range of clinically important bacteria [36-38].

Using SMRT sequencing technology, we determined the full context of *bla*$_{IMP-4}$ and its location within a large, complex and highly repetitive MDR region harbouring two integrons: In37 and In809. In37 is a widespread class 1 integron that has been found in many bacterial species [39, 40]. In809, which carries *bla*$_{IMP-4}$, has previously been described from *Klebsiella pneumoniae* (GenBank: KF250428.1, HQ419285.1, AJ609296.3), *E. cloacae* (GenBank: JX101693.1) and *Acinetobacter baumannii* (GenBank: AF445082.1, DQ532122.1) in various plasmid backgrounds including IncA/C2 [41], IncL/M and IncF [42]. Most recently, a carbapenemase-producing *Salmonella* sp. isolated from a domestic cat in Australia was shown to contain *bla*$_{IMP-4}$ within an IncHI2 MDR plasmid (pIMP4-SEM1) [43]. Remarkably, we found that pIMP4-SEM1 was near identical to pMS7884A (Figure S5). This finding highlights the role of domestic animals (or the food they eat) as a reservoir for antibiotic resistance genes.

Analysis of several CPE in this study suggested that a common plasmid or integron carrying multiple antibiotic resistance genes is likely the major driver of antibiotic resistance dissemination across a broad range of Enterobacteriaceae. In addition to the presence of *bla*$_{IMP-4}$, four resistance genes (*bla*$_{TEM-1b}$, *bla*$_{IMP-4}$, *qnrB*, and *aac(6')-Ib*) carried by these isolates were previously detected by PCR in the majority of 29 IMP-4-producing *E. cloacae* isolates surveyed from Queensland hospitals between June 2009 to March 2014 [13]. Only one of these isolates was ST90, suggesting lateral transfer of these genes to different *Enterobacter* clones in Queensland before 2013.

There were significant discrepancies between meropenem MICs according to the testing modality used, with the Etest consistently testing as "susceptible/intermediate" (MIC ≤4 mg/L; range 0.5-4 mg/L) and Vitek2 as "resistant" (usually with MICs ≥16 mg/L). According to pharmacokinetic/pharmacodynamic (PK/PD) principles, provided the MIC to a carbapenem falls within a susceptible range, the agent may still be effective despite the presence of a carbapenemase [44]. Robust clinical data to help guide therapy are lacking and many clinicians rely on combination

therapy to optimize efficacy against carbapenemase-producers, largely based on observational studies suggesting benefit [45, 46]. The presence of carbapenemase genes may be missed if clinical breakpoints for carbapenem MICs are used [30], however it can be rapidly ascertained by WGS, without *a priori* assumptions of which genes are likely to be present. A wealth of additional information that may influence clinical decisions can be obtained, such as the presence of other β-lactamases, factors that may regulate resistance gene expression (e.g. IS elements), mutations in outer-membrane proteins, or other known resistance genes.

Conclusions

We used WGS to help elucidate genetic relationships between $bla_{\text{IMP-4}}$ carbapenemase-producing *E. cloacae* identified from our ICU and Burns facility. Real-time application of this technology revealed an unexpected clonal relationship with a strain isolated from the same unit two years previously. Comparison with other Enterobacteriaceae containing $bla_{\text{IMP-4}}$ isolated from surrounding hospitals revealed its carriage on a broad host range IncHI2 plasmid, assumed to be circulating via lateral gene transfer across different *E. cloacae* clones and also *E. coli*. SMRT sequencing enabled the genetic context of all resistance genes within this plasmid to be resolved and revealed the mechanism of loss of resistance genes in one *E. cloacae* strain that reverted to a fully carbapenem-susceptible phenotype. As WGS technologies become increasingly available, they are likely to prove an essential tool for the clinical microbiology laboratory to respond to emergent infection control threats, and can be used in real-time to provide clinically meaningful information.

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Table 1: Antibiotic Resistance Profile as determined by Etest, Vitek2 and ResFinder

Patient			1		2				3			
Strain			7884	7885	7886	7887	7888	7889	7890	7891	7892	7893
Source			ETT	urine	ETT	urine	blood	urine	ETT	blood	Leg swab	blood
ST			90	90	90	90	90	90	90	90	90	90
Plasmid			IncHI2	IncHI2	IncHI2	IncHI2	IncHI2	IncHI2	IncHI2	IncHI2	IncHI2	IncHI2
MIC(mg/L) by E-test		Ertapenem	1	2	4	2	0.5	0.032	2	0.5	0.5	2
		Imipenem	2	1	4	8	1	0.5	2	1	1	4
		Meropenem	0.5	1	4	2	0.5	0.032	2	1	0.5	0.5
β-lactams and Cephalosporins	Vitek2[1]	Tim	≥128	≥128	≥128	≥128	≥128	32	≥128	≥128	≥128	≥128
		Mer	≥16	≥16	≥16	≥16	≥16	≤0.25	≥16	≥16	≥16	≥16
		Taz	16	16	16	16	16	8	16	16	16	16
		Fox	≥64	≥64	≥64	≥64	≥64	≥64	≥64	≥64	≥64	≥64
		Caz	≥64	≥64	≥64	≥64	≥64	≤1	≥64	≥64	≥64	≥64
		Cro	16	16	16	16	16	≤1	16	16	16	8
		Fep	2	2	4	2	2	≤1	2	2	2	4
	Res	*ampC*	+	+	+	+	+	+	+	+	+	+
		bla_{OXA-1}	+	+	+	+	+	-	+	+	+	+
		bla_{IMP-4}	+	+	+	+	+	-	+	+	+	+
		bla_{TEM-1B}	+	+	+	+	+	+	+	+	+	+
Aminoglycosides	Vitek2	Ami	≤2	≤2	≤2	≤2	≤2	8	≤2	≤2	≤2	≤2
		Gent	≥16	≥16	≥16	≥16	≥16	≤1	≥16	≥16	≥16	≥16
		Tob	8	8	8	8	8	≥16	8	8	8	8
	Res	*strB*	+	+	+	+	+	+	+	+	+	+
		strA	+	+	+	+	+	+	+	+	+	+
		aac(6')Ib-cr	+	+	+	+	+	+	+	+	+	+
		aac(3)-IId	+	+	+	+	+	-	+	+	+	+
Quinolones	Vitek2	Cip	≤0.25	0.5	≤0.25	≤0.25	0.5	≤0.25	0.5	0.5	1	≤0.25
		Nor	2	2	2	2	2	0.5	2	2	2	1
	Res	*qnrB2*	+	+	+	+	+	-	+	+	+	+
Sulphonamide/ Trimethoprim	Vitek2	Tmp/smx	≥320	≥320	≥320	≥320	≥320	≥320	≥320	≥320	≥320	≥320
	Res	*sulI*	+	+	+	+	+	+	+	+	+	+
		dfrA18	+	+	+	+	+	+	+	+	+	+
Rifampicin	Res	*arr3*	+	+	+	+	+	-	+	+	+	+
Macrolide	Res	*mph(A)*	+	+	+	+	+	-	+	+	+	+
Phenicols	Res	*catA2*	+	+	+	+	+	+	+	+	+	+
		catB3	+	+	+	+	+	+	+	+	+	+
Tetracycline	Res	*tet(D)*	+	+	+	+	+	+	+	+	+	+

[1]Res = ResFinder Antimicrobial Resistance gene database; Vitek = Vitek2 automated susceptibility

MIC (mg/L): Tim=ticarcillin-clavulanate, Taz=piperacillin-tazobactam, Fox=cefoxitin, Caz=ceftazidime, Cro=ceftriaxone, Fep=cefepime, Mer=meropenem, Ami=amikacin, Gent=gentamicin, Tob=tobramycin, Cip=ciprofloxacin, Nor=norfloxacin, Tmp/smx=trimethoprim-sulphamethoxazole

490 **Figure Legends:**

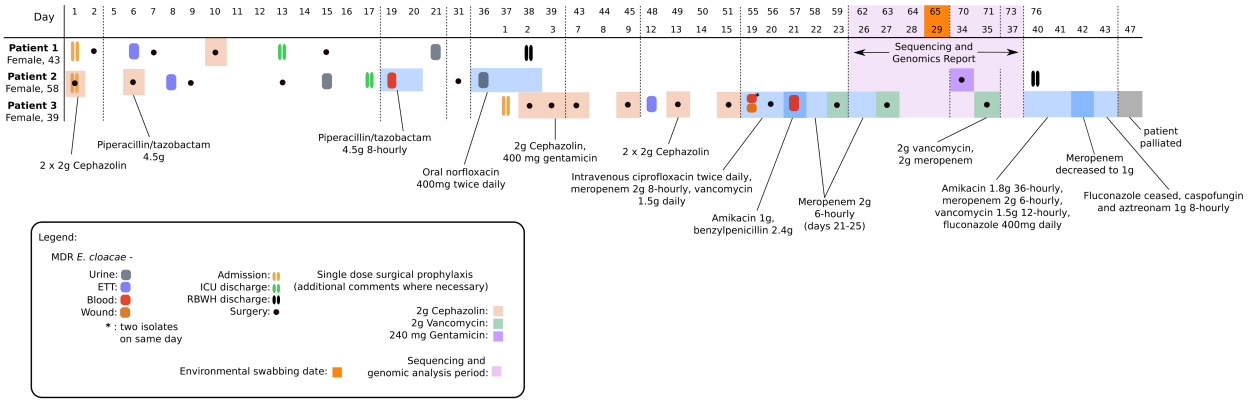
491 **Figure 1: RBWH clinical case study outline**: Three burns patients were admitted to the RBWH
492 ICU ward in mid 2015. Patient 1 (Female, 43-years-old) and Patient 2 (Female, 58-years-old) were
493 admitted on the same day. Subsequent to admission, both patients developed carbapenem-resistant
494 *E. cloacae* infections, with two samples taken from patient 1 (source = ETT [purple] and urine
495 [grey]), and 4 samples taken from patient 2 (source = ETT [purple], urine [grey], and blood [red]).
496 Patient 3 (Female, 39-years-old) was admitted 37 days after the patient 1 and 2 had been admitted
497 and after they had been discharged from the ICU. Patient 3 also developed infection due to a
498 carbapenem-resistant *E. cloacae* infection, and had 4 samples taken from ETT (purple), blood (red)
499 and wound sites (orange). After intensive antibiotic and antifungal treatment, the patient was
500 palliated on day 47 of ICU admission. Sequencing and genomics analysis of all 10 isolates was
501 undertaken following confirmation of all three patients being infected with $bla_{\text{IMP-4}}$-producing *E.*
502 *cloacae* (period shown in purple shading). Environmental swabbing was undertaken 65 days after
503 the initial admission of patient 1 and 2, and 29 days after the admission of patient 3 (orange square).
504

505 **Figure 2: CRE isolate timeline and relationship matrix: A.** 10 isolates were collected from 3
506 patients at various time-points in mid 2015. Coloured blocks indicate the source of the isolated
507 strain: purple: respiratory, grey: urine, red: blood, and orange: wound. **B.** Relationship matrix (left)
508 shows specific core single nucleotide variant (SNV) differences identified between strains. Strains
509 within the same circle have identical core SNV profiles. Lines connecting circles represent
510 accumulating SNV differences between strains (not-to-scale), where each line represents one SNV
511 (including nucleotide deletion). Specific nucleotide differences between isolates are given in the
512 table in panel B. Locations and consequences of nucleotide change are shown in Supplementary
513 Dataset S1. All 11 isolates differed by 5 SNVs overall.
514

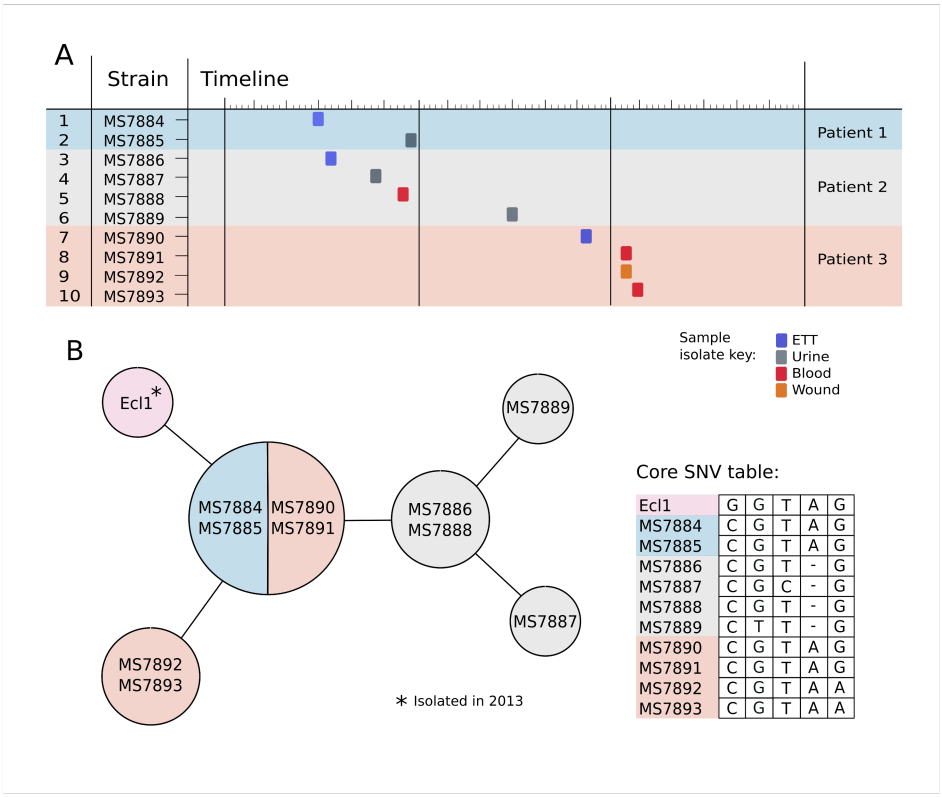
Figure 3: Core SNP Maximum likelihood (ML) tree of Hospital A and B *E. cloacae* isolates in relation to RBWH isolates: Trimmed reads from 6 *E. cloacae* isolates (Hospital A and B) were aligned to the reference *E. cloacae* Ecl1 (isolated in 2013 at the RBWH) to determine core single nucleotide polymorphisms (SNPs) between all isolates. Ecl1 in this figure represents all 2015 RBWH isolates (n=10) as they were found to be near identical at the core genome level. 63,861 core SNPs were identified and used to generate a ML tree with RAxML (1000 bootstrap replicates), which determined no relationship between the RBWH isolates (pink) and the Hospital B (blue)/Hospital A (orange) isolates. Four closely related strains were identified from Hospitals A and B (red box). Alignment of trimmed reads from MS8077, MS8079 and MS7926 to MS7924 identified 117 core SNPs, however, a number of these SNPs were removed as they were identified as residing within transposon or phage regions. The remaining 58 core SNPs were used to generate a ML tree (1000 bootstrap replicates), showing that Hospital B strains differ by less than 20 SNPs.

Figure 4: Large IncHI2 plasmid with ~55 kb multidrug resistance region containing IMP-4 carbapenemase: A. A 330,060 bp IncHI2 plasmid carrying multiple resistance operons, including a large ~55 kb multidrug resistance (MDR) region, was fully recovered and assembled using Pacific Biosciences (PacBio) SMRT sequencing of strain MS7884 (patient 1, isolate 1). The multidrug resistance region was found to contain two class 1 integrons (In809, In37) along with several other antibiotic resistance genes, as indicated. Comparison of this MDR region to publicly available genomes found a close match to pEl1573, isolated in 2012 from an *E. cloacae* isolate in Sydney, Australia. **B.** A predicted model of homologous recombination between two *aac(6')-Ib* (aac6) genes (red asterisks) within the ~55 kb MDR region in MS7889 (patient 2, isolate 4, IMP-, carbapenem-susceptible) leading to the loss of a ~34 kb region containing $bla_{\text{IMP-4}}$ as well as several other antibiotic resistance genes.

Figure 1



543 **Figure 2**



544
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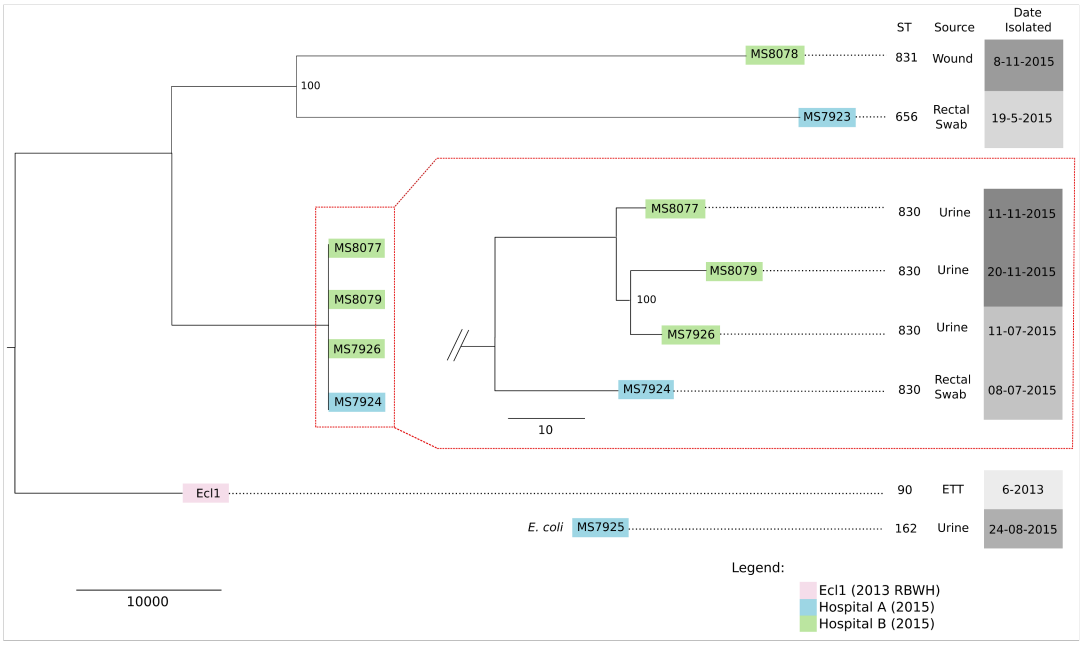
546 **Figure 3**



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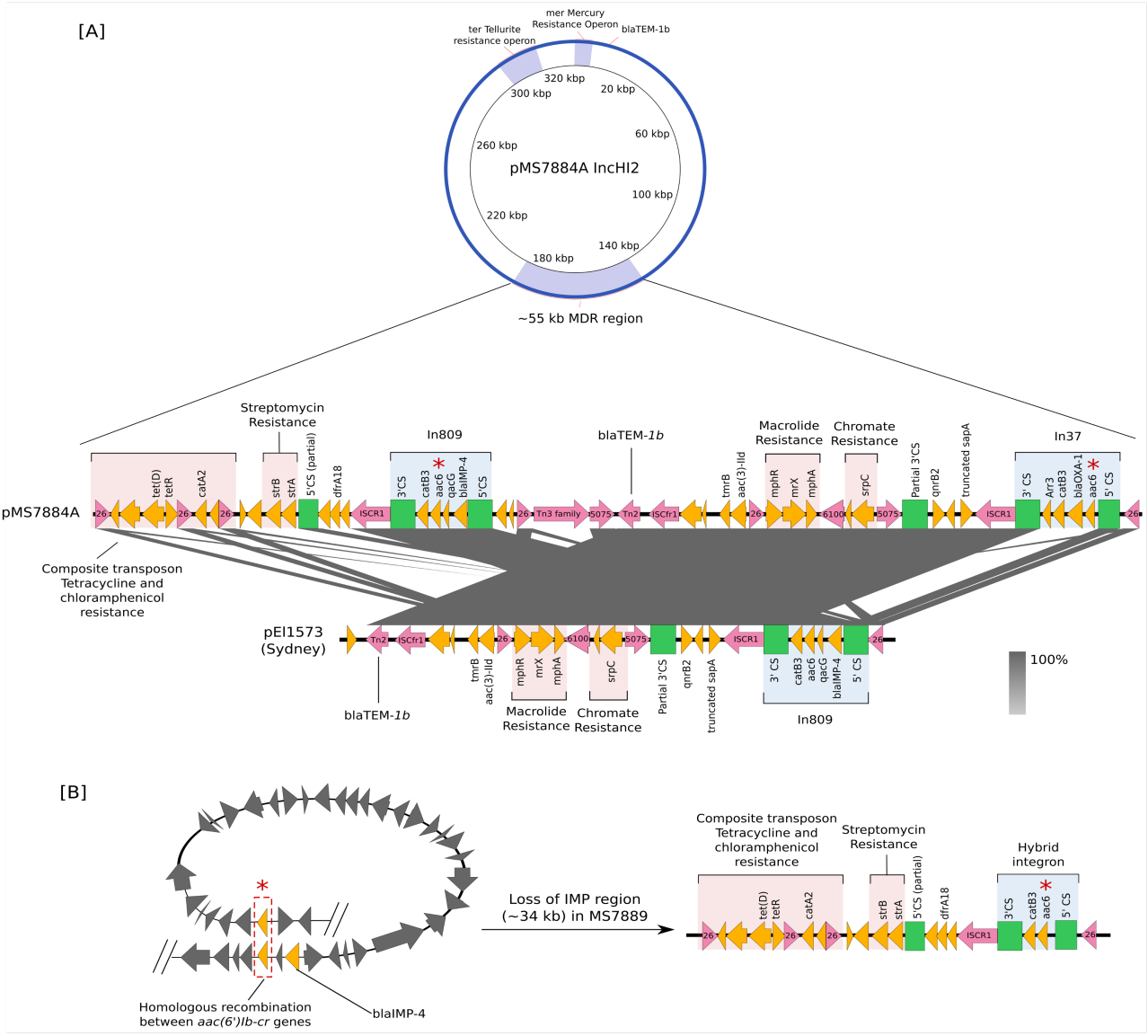
549 **Figure 4**



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