Using whole-genome sequencing and a pentaplex real-time PCR to characterize third-generation cephalosporin-resistant Enterobacteriaceae from Southeast Queensland, Australia

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Abstract

Third-generation cephalosporin-resistant (3GC-R) Enterobacteriaceae represent a major threat to human health. Here, we captured 288 3GC-R Enterobacteriaceae clinical isolates from 258 patients presenting at a regional Australian hospital over a 14-month period. Alongside routine mass spectrometry speciation and antibiotic sensitivity testing, isolates were examined using a rapid (~40 min) pentaplex real-time PCR assay targeting the most common extended spectrum β-lactamases (ESBLs; CTX-M-1 and CTX-M-9 groups, plus TEM, SHV, and an internal 16S ribosomal DNA control). Additionally, AmpC CMY β-lactamase prevalence was examined using a singleplex PCR. A subset of isolates, including all 3GC-R isolates obtained from the intensive care unit, were subjected to whole-genome sequencing (WGS) to assess transmission dynamics, the presence of unidentified resistance determinants, and genotyping accuracy. *Escherichia coli* (80.2%) and *Klebsiella pneumoniae* (17.0%) were dominant, with *Klebsiella oxytoca*, *Klebsiella aerogenes* and *Enterobacter cloacae* infrequently identified. Ceftriaxone and cefoxitin resistance was identified in 97% and 24.5% of *E. coli* and *K. pneumoniae* isolates, respectively. Consistent with global findings in Enterobacteriaceae, the majority (98.3%) of isolates harbored at least one β-lactamase gene, with 144 (50%) encoding $bla_{\text{CTX-M-1}}$ group, 92 (31.9%) $bla_{\text{CTX-M-9}}$ group, 48 (16.7%) bla_{SHV}, 133 (46.2%) bla_{TEM}, and 34 (11.8%) bla_{CMY} genes. WGS of β-lactamase negative or carbapenem-resistant isolates identified uncommon ESBLs and carbapenemases, including bla_{NDM} and bla_{IMP}, and confirmed all PCR-positive genotypes. No evidence of transmission among intensive care unit patients was identified. We demonstrate that our PCR assays enable the rapid and cost-effective identification of ESBLs in the hospital setting, which has important infection control and therapeutic implications.

Introduction

Third-generation cephalosporin resistant (3GC-R) Enterobacteriaceae represent a significant threat to human health due to their ability to rapidly transmit antimicrobial resistance (AMR) determinants within and among bacterial populations [1]. Both infection and colonization with these globally-disseminated organisms are associated with poor clinical outcomes and death [2]. 3GC-R Enterobacteriaceae prevalence rates vary considerably among hospitals and countries, with particularly high rates in India, Asia, and the Middle East [3]. Australia has historically observed a relatively low frequency of 3GC-R Enterobacteriaceae. However, this pattern may be changing, with an extended spectrum β-lactamase (ESBL) phenotype detected in 13.3% of *E. coli* and 9.8% of *K. pneumoniae* in blood culture isolates in 2018 [4], up from 2013 rates of 7.6% and 6.3%, respectively. Moreover, ceftriaxone resistance was seen in 13.4% of *E. coli* and 9.4% of *K. pneumoniae*, with 86.3% and 82.6% containing CTX-M-type ESBL genes, respectively [4].

AMR in 3GC-R Enterobacteriaceae is generally conferred by the presence of an ESBL or the over-expression of a chromosomal or plasmid-borne AmpC β-lactamase; however, the prevalence of these determinants vary greatly depending on the geographic region under examination [5]. CTX-M-type ESBLs are currently the most common AMR determinants found in 3GC-R Enterobacteriaceae isolated in Australian hospitals, with CTX-M-1 and CTX-M-9 groups collectively comprising 86.2% of identified CTX-M-type enzymes [4]. Detection of ESBL subtypes is not standard clinical practice; instead, their presence is typically inferred from antimicrobial susceptibility patterns and phenotypic confirmatory tests, which are laborious, costly (~$AUD25/isolate), and time-consuming (>24h) to perform [6]. Use of rapid, accurate, and inexpensive molecular-based diagnostics for AMR determinant detection in the clinical setting may improve patient outcomes by enabling more rapid, directed, and personalized treatment strategies, thereby improving antibiotic stewardship measures and

reducing AMR burden. This utility has already been demonstrated in a number of clinical settings including for bloodstream infections, pneumonia, and infection control screening [7-9].

The implementation of whole-genome sequencing (WGS) into clinical practice is rapidly becoming a reality in many parts of the world. In the infectious diseases field, WGS enables the precise identification of AMR determinants in clinical samples (including polymicrobial specimens), and the inference of population structure and transmission dynamics on a local (e.g. single hospital) through to a global scale. Multiple studies have used WGS to better understand Enterobacteriaceae population structure in the hospital setting [10, 11], often in an effort to better understand transmission dynamics and to control nosocomial infection spread. These studies have provided valuable insights into the population structure of the Enterobacteriaceae, including the identification of a rapid clonal expansion of *E. coli* sequence type 131 (ST131) [12, 13] and an increased appreciation for the high rates of AMR in *K. pneumoniae*, which is now recognized as a significant global threat [3].

In the current study, we prospectively collected all 3GC-R Enterobacteriaceae identified over a 14-month period at the Sunshine Coast University Hospital, Queensland, Australia, a 450-bed, regional teaching and tertiary hospital. Using large-scale comparative genomics, we developed a novel pentaplex assay and a bla_{CMY} singleplex assay to rapidly detect the most commonly identified β-lactamase (including ESBL) genes in 3GC-R Enterobacteriaceae. In association with WGS, we used our assays to determine the molecular epidemiology of the β-lactamase genes in our sample set and compared the accuracy of our assays to both genotypic and phenotypic typing methods to validate accuracy, sensitivity and specificity. Additionally, we determined the population structure and transmission dynamics of our

101 3GC-R Enterobacteriaceae population to identify potential cases of nosocomial spread or

102 outbreak clusters.

Methods

Ethics. This study was approved by The Prince Charles Hospital Human Resources Ethics Committee (HREC/18/QPCH/110). A waiver of informed consent was granted due to the low to negligible risk associated with this study.

Sample collection and clinical information. Clinical specimens from 258 adult and pediatric patients culturing 3GC-R Enterobacteriaceae were collected prospectively from July 2017 through September 2018 at the Sunshine Coast University Hospital microbiology laboratory (*n*=288). Our patient cohort was consistent with other studies, being predominantly female (64.0%) and aged over 65 (60.9%), with an age range of 2 to 98 years old. Intensive care unit (ICU) patient specimens comprised 54 (18.8%) of the total collection, and were obtained from 48 ICU patients over a 12-month period. From the ICU cases, 37 *E. coli*, 9 *K. pneumoniae,* two *K. oxytoca* and one *E. cloacae* were identified from rectal swabs (*n*=49; 90.7%) with urine, sputum and pus making up the remaining five samples. Of the non-ICU patients (*n*=216 with 234 samples), the breakdown of clinical samples included urine (*n*=154; 65.8%), rectal swabs (*n*=66; 28.2%), blood (*n*=7; 3%) and pus (*n*=6; 2.6%). Isolates were stored in Luria Bertani broth containing 20% glycerol at -80^{o}C. Patient and hospital epidemiological data were also documented and linked to each specimen. Once collected, isolates were speciated using the VITEK® 2 MALDI-TOF MS with the GN ID card (bioMérieux, Murrarie, QLD, Australia). Isolate information is detailed in Table S1.

Susceptibility testing. Antibiotic susceptibility testing and minimum inhibitory concentration (MIC) values were determined using VITEK® 2 or Etest (bioMérieux) according to the manufacturer's instructions. EUCAST criteria (v.10.0) were used for susceptibility and resistance interpretation.

DNA preparation. For PCR, crude DNA was extracted from all isolates using 5% chelex-100 resin (Bio-Rad Laboratories, Gladesville, NSW, Australia) in 96-well plate format. Due to the potential for cross-contamination, approximately six no-template controls were randomly included on each DNA plate. A small toothpick-sized scraping of culture was suspended into the chelex resin, followed by plate sealing and heating to 95°C for 10 min in a PCR thermocycler. Following centrifugation at 4,000 x *g* for 2 min, a 1:10 dilution of DNA was prepared in molecular-grade H_2O (Sigma-Aldrich, Castle Hill, NSW, Australia); this dilution was used for PCR. For a subset of samples (*n*=50), a pinhead-sized amount of culture collected using a sterile 10 µL pipette tip was placed directly into 4 µL of PCR mastermix to facilitate a more rapid turnaround time. For WGS, DNA was extracted using the Quick-DNA miniprep kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions.

PCR development and β-lactamase detection. A pentaplex real-time PCR assay was developed to detect the two major CTX-M ESBL groups ($bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-9}}$), bla_{SHV} and bla_{TEM} (designed to amplify both ESBL and non-ESBL bla_{SHV} and bla_{TEM}), and all variants of bla_{CMY} β-lactamase genes (Figure 1). To identify conserved regions for assay design, all publicly available genetic sequences for $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-9}}$, bla_{CMY}, bla_{SHV}, and bla_{TEM} were downloaded from the NCBI Nucleotide database (as at March 2019) and aligned using Clustal Omega v1.2.2. Aligned sequences were visualized in Geneious v11.0.4. Candidate primers and probes were identified in Primer Express v3.0 (Applied Biosystems, Foster City, California, USA) or Primer3 v0.4.0 [14] followed by primer dimer and heterodimer assessment using NetPrimer (http://www.premierbiosoft.com/netprimer/).

For the pentaplex assay, Black Hole Quencher probes were used to enable multiplexing of five assays in a single PCR well. The following primers and probes (5' to 3'; Biosearch

Technologies, Novato, CA, USA; Macrogen Inc., Geumcheon-gu, Seoul, Republic of Korea) were designed: CTX-1 (CTX1_F: CGAMACGTTCCGTCTCGAC, CTX1_R: CTTTCATCCAYGTCACCAGCTG, CTX1_P1: FAM-CGTGATACCACTTCACCTCGGG-BHQ1), CTX-9 (CTX9_F: CGTCSCGCTCATCGATACC, CTX9_R: CAGCTGCTTTTGCGTTTCACTC, CTX9_P1: HEX-CGCTTTCCAATGTGCAGTACC-BHQ1), SHV (SHV_F: CCARCGTCTGAGCGCC, SHV_R: TATCGGCGATAAACCAGCCC, SHV_P1: Cy5-CAGCACGGAGCGGATCA-BHQ2), TEM (TEM_F: TGTGGYGCGGTATTATCCCGT, TEM_R: TGCATAATTCTCTTACTGTCAWGCCA, TEM_P1: Cy5.5-CACCAGTCACAGAAAAGCATCT-BHQ2), and a previously described 16S assay [15] using a different fluorophore on the probe (Texas Red-CACGAGCTGACGACARCCATGCA-BHQ2) to accommodate a pentaplex format. Last, an additional assay targeting CMY was included as a singleplex assay: CMY (CMY_F: TGGCGTATTGGYGATATGTA, CMY_R: TTATGCACCCATGAGGCTTT, CMY_Pr: FAM-TGGGAGATGCTGAACTGGCC-BHQ1). Coincidentally, the reverse primer sequence for our CMY assay was identical to one designed previously [16].

PCRs were carried out using 0.1 µM of the 16S probe, 0.2 µM of the 16S primers, TEM primers and the CTX-1, CTX-9, and TEM probes, 0.3 µM of the SHV primers, and 0.4 µM of the CTX-1, CTX-9, and CMY primers, and the SHV and CMY probes. Reactions contained 1X Sso Advanced Universal Probes Supermix (Bio-Rad), primers, probes, 1 µL DNA template, and molecular grade H_2O to a 5 µL total reaction volume. PCRs were performed on a CFX96 Touch Real-Time PCR Detection System using white Hard Shell 96-well PCR plates and Microseal 'B' Adhesive seals (Bio-Rad) or 0.2 mL 8-tube PCR strips (Bio-Rad). Thermocycling conditions comprised an initial 2 min denaturation at 95 °C, followed by 45 cycles of 95 °C for 1 sec and 60 °C for 3 sec (total run time of 39 min). Due to the inclusion of the 16S rDNA internal control, each sample were run as a single reaction, with any PCR failures according to 16S (either due to no amplification, or amplification >25 cycles) repeated as required.

Whole-genome sequencing (WGS). WGS was performed at the Australian Centre of Ecogenomics (University of Queensland, St Lucia, QLD, Australia) using the metagenomics sequencing protocol on the NextSeq 500 instrument (Illumina, San Diego, CA, USA) with libraries constructed using Nextera DNA Flex (Illumina).

Genomic analysis. All genomic data were lightly quality-filtered using Trimmomatic v0.39 [17] using parameters described elsewhere [18]. Following quality filtering, genomes for each sample were screened for the presence of multiple species using Kraken 2 v2.0.8 [19]. Single-species genomes were assembled using MGAP v1.1 (https://github.com/dsarov/MGAP---Microbial-Genome-Assembler-Pipeline), whereas genomes containing multiple species were assembled with metaSPAdes [20]. Assembled contigs were binned with MaxBin v2.0 [21] and speciated with Kraken 2 v2.0.8 [19]. Antibiotic resistance genes were identified through the Resistance Gene Identifier tool within the Comprehensive Antimicrobial Resistance Database (CARD) [22]. Phylogenomic analysis was performed on biallelic, orthologous, core-genome single-nucleotide polymorphisms (SNPs) using SPANDx v3.2.1 [23]. Samples identified as containing multiple strains according to the method described by Aziz and colleagues [24] were excluded from phylogenetic analysis (*n*=7). Samples containing multiple species were split using the procedure described above and included in phylogenetic analysis (*n*=4). *E. coli* O157:H7 Sakai [25] or *K. pneumoniae* HS11286 [26] were used as read mapping reference genomes for species-specific analyses. To achieve the highest possible resolution, error-corrected assemblies of strains with matching multilocus sequence types (STs) were used as reference genomes for transmission analyses. ST profiles were determined using SRST2 v0.2.0 [27]. Phylogenetic trees were reconstructed using the heuristic search feature of PAUP v4.4.7 [28] and visualized in iTOL v5.5[29].

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208 **WGS data.** Raw sequence reads are available on the NCBI Sequence Read Archive database

209 under BioProject accession number PRJNA606985.

Results and Discussion

ESBL enzymes have established themselves as major contributors of AMR in Gram-negative bacteria and are increasing in prevalence, particularly in the community setting. Over the course of 14 months, we collected isolates from all diagnosed cases of 3GC-R Enterobacteriaceae in a regional hospital in Southeast Queensland, Australia. Among the 288 Gram-negative isolates collected during this study, *E. coli* and *K. pneumoniae* were dominant, comprising 80.2% and 17.0% of the isolates, respectively (Table 1). The remaining isolates were identified as *E. cloacae* (1.4%; *n*=4), *K. oxytoca* (1.0%; *n*=3), and *K. aerogenes* (0.3%; *n*=1). Resistance to ceftriaxone, ceftazidime and cefepime was common and observed in 96.2%, 93.4%, and 86.8% of isolates, respectively, whereas cefoxitin resistance was noted in only 81 (28.1%) isolates (Table 1). Meropenem resistance was seldom observed, with only six strains (2.1%) exhibiting resistance to this carbapenem. The double disc diffusion method for ESBL phenotypic testing was negative in 32 (11.1%) isolates, indicating the presence of a 3GC-R mechanism with retention of a resistant phenotype in the presence of a β-lactamase inhibitor (e.g. clavulanic acid), likely due to over-expression of an AmpC β-lactamase. The remaining 256 (88.9%) isolates were positive according to the double disc diffusion method, exhibiting sensitivity in the presence of a β-lactamase inhibitor.

Redesign of current ESBL PCR assays to improve specificity and detection rates. We next set out to identify the abundance of 3GC-R AMR determinants in our isolate collection using real-time PCR. The rapid expansion of WGS data in recent years has enabled improvements to be made upon assays designed in the pre-WGS era, which risk being either non-specific or intolerant to sequence diversity within enzyme families. Given a sufficiently large and diverse dataset, sequence diversity can result in a non-trivial proportion of false-negative calls, skewing epidemiological observations. Examination of previously published ESBL assays [30] identified several mismatches in primer/probe pairs, with the mismatches especially

problematic for the CTX-M enzymes (data not shown). To overcome issues with false-negative results using existing assays, we analyzed the extensive volume of publicly available sequence data to identify conserved sequence regions suitable for assay design. Our redesigned assays target the genes encoding the most common ESBLs (CTX-M group 1, CTX-M group 9), along with SHV and TEM β-lactamases, which can evolve to become ESBLs through mutations in their active sites or contribute to clinically relevant AMR due to increased expression.

ESBL and ß-lactamase prevalence in 3GC-R Enterobacteriaceae. Preliminary validation of our newly designed PCR assays was performed to determine their accuracy and specificity. The assays were first tested on a characterized set of 12 strains (Table S2) that have previously been subjected to WGS [31]. Our assays showed 100% sensitivity and specificity for all enzymes (CTX-M group 1, CTX-M group 9, CMY, SHV and TEM) according to corresponding WGS data. Following this confirmation, our assays were run across the entire isolate set (*n*=288) in pentaplex format (*bla*$_{\text{CTX-M-1}}$ group, *bla*$_{\text{CTX-M-9}}$ group, *bla*$_{\text{SHV}}$, *bla*$_{\text{TEM}}$, 16S rDNA; Figure 1), with *bla*$_{\text{CMY}}$ tested in singleplex format. Our assays performed well, with 100% concordance between PCR genotypes and WGS of 98 isolates, and no false-negatives identified. Overall, a β-lactamase genotype was identified in 277 (96.2%) isolates using PCR. Consistent with the previously reported prevalence of 3GC-R enzymes in Australia and New Zealand [10], we found that *bla*$_{\text{CTX-M-1}}$ group was most common, being present in 144/288 (50%) isolates, including 105/231 (45.5%) *E. coli* and 36/49 (73.5%) *K. pneumoniae* isolates. The *bla*$_{\text{CTX-M-9}}$ group was next most common, being found in 93/288 (32.3%) isolates, including 89/231 (38.5%) *E. coli* and 4/49 (8.2%) *K. pneumoniae* isolates. The presence of both *bla*$_{\text{CTX-M-1}}$ group and *bla*$_{\text{CTX-M-9}}$ group genes was identified in five (1.7%) *E. coli* and one *K. pneumoniae* isolate. Of the eight isolates belonging to other species, *bla*$_{\text{CTX-M-1}}$ and *bla*$_{\text{CTX-M-9}}$ group genes were present in 4/8 (50%) and 0/8 (0%) of these isolates, respectively. The

acquired *ampC* gene, *bla*$_{CMY}$, was identified in 30/231 (13%) of *E. coli*, 3/49 (6.1%) of *K. pneumoniae*, and 1/8 (13%) other Enterobacteriaceae species. *bla*$_{CMY}$ and *bla*$_{CTX-M-1}$ were both present in seven isolates (five *E. coli*, one *K. pneumoniae*, and one *E. cloacae*) and *bla*$_{CMY}$ and *bla*$_{CTX-M-9}$ were present in two *E. coli* isolates only (Table 1).

Seven strains (five *E. coli* and two *K. pneumoniae*) have *bla*$_{SHV}$ only; nine *E. coli* have *bla*$_{TEM}$ only, and seven (five *K. pneumoniae*, one *E. coli*, and one *E. cloacae*) have both *bla*$_{SHV}$ and *bla*$_{TEM}$. Although most SHV (e.g. *bla*$_{SHV-1}$) and TEM (e.g. *bla*$_{TEM-1}$, *bla*$_{TEM-2}$) enzymes do not typically cause 3GC-R, their presence in strains that lack CTX-M-type ESBLs should be investigated, as these enzymes can occasionally confer 3GC-R through active site mutation or overexpression [5]. Of the 14 strains encoding *bla*$_{SHV}$ but not *bla*$_{CTX-M-1}$ group, *bla*$_{CTX-M-9}$ group, or *bla*$_{CMY}$ enzymes, five had accompanying WGS data, which permitted assessment of *bla*$_{SHV}$ ESBL status. In all cases, the *bla*$_{SHV}$ variant was an ESBL (two *bla*$_{SHV-2}$, one *bla*$_{SHV-64}$, one *bla*$_{SHV-66}$, and one *bla*$_{SHV-134}$) [32]. In contrast, none of the four strains encoding *bla*$_{TEM}$ but not *bla*$_{CTX-M-1}$ group, *bla*$_{CTX-M-9}$ group, *bla*$_{CMY}$, or ESBL-encoding *bla*$_{SHV}$ enzymes were ESBLs, with all being *bla*$_{TEM-1}$. Three of these were negative with the double disc diffusion method, indicating probable chromosomal mutations underpinning this phenotype. The basis for the ESBL phenotype in the fourth strain could not be elucidated.

Due to the conserved nature of our TEM and SHV assays to detect all enzyme variants, we do not recommend these assays for identifying ESBL-conferring mutations in *bla*$_{TEM}$ or *bla*$_{SHV}$; rather, they can only be used to guide further diagnostic tests. However, the quantitative nature of our pentaplex assay, which includes an internal 16S rDNA control, permits the detection of copy number increases in the β-lactamase genes. By examining the cycles-to threshold differences between 16S and *bla*$_{TEM}$ and *bla*$_{SHV}$, we identified an ~8-10x copy number increase in SCHI0016.S.139, which also possessed *bla*$_{CTX-M-15}$. Consistent with this

PCR result, WGS verified that this strain harbored a multicopy plasmid carrying *bla*$_{\text{TEM-135}}$. TEM-135 differs from TEM-1 by having a M182T mutation that confers improved β - lactamase stability and potential for third-generation cephalosporin hydrolysis and ESBL activity [33]. Therefore, examining β-lactamase copy number is important when determining ESBL potential from genetic or genomic data, particularly in strains where common ESBLs are not identified.

Correlation of bla$_{\text{CMY}}$ *and cefoxitin resistance*. Of the 81 isolates resistant to cefoxitin by phenotypic methods, *bla*$_{\text{CMY}}$ was detected in only 31 (38.3%) cases. Resistance to cefoxitin is often used as a surrogate marker for AmpC presence, although this approach is imperfect [34]. This correlation is also dependent on the MIC, with higher MICs more reliably predicting AmpC β-lactamase presence [34]. Our findings concur with this prior work, with 61.7% of isolates failing to amplify the *bla*$_{\text{CMY}}$ target, suggesting a lack of sensitivity in using cefoxitin as a screening tool for AmpC enzymes. In support of this hypothesis, AmpC has previously been reported as an underappreciated mechanism of 3GC-R due to a lack of standardized testing for its detection [35]. As such, molecular methods such as PCR that can detect other common and emerging acquired *ampC* gene families (e.g. *bla*$_{\text{DHA}}$, *bla*$_{\text{OXY}}$, *bla*$_{\text{MIR}}$) may prove useful for infection control management and treatment of multidrug-resistant Gram-negative infections. This is particularly true in the case of piperacillin-tazobactam, which may not be a stable or reliable antibiotic against AmpC producers [10, 36].

Whole-genome sequencing analysis identifies uncommon ESBL genes. Eleven (3.8%) isolates negative with all PCR assays except the 16S rDNA internal control (seven *E. coli*, one *K. pneumoniae*, one *K. oxytoca*, one *K. aerogenes* and one *E. cloacae*) were subjected to WGS to determine the molecular basis for their 3GC-R phenotype. Six were found to carry chromosomal AmpC enzymes, with three *E. coli* encoding *bla*$_{\text{DHA-1}}$, one *K. pneumoniae/K.*

oxytoca mixture harboring $bla_{OXY-2-4}$, one *K. oxytoca* carrying $bla_{OXY-2-1}$, and one *E. cloacae* carrying bla_{ACT-17}. The remaining five isolates had no discernable 3GC-R determinants, although misclassification via automated susceptibility testing or upregulation of chromosomal AmpC β-lactamases are both possible causes for this discrepancy.

Six isolates (three *E. cloacae*, two *E. coli* and one *K. pneumoniae*) exhibiting meropenem resistance alongside 3GC-R were also subjected to WGS to determine the basis of resistance towards this carbapenem. As expected, meropenem resistance was mostly conferred by carbapenemases, with 4/6 (66.6%) isolates possessing one of these enzymes. bla_{NDM-1} or bla_{IMP-4} were identified in two *E. cloacae* isolates, bla_{NDM-5} was identified in an *E. coli* strain, and an OXA-48-like enzyme, $bla_{OXA-232}$, was identified in a *K. pneumoniae* isolate. No carbapenemase gene could be identified in the remaining two isolates (one *E. coli* and one *E. cloacae*); although not investigated in this study, chromosomally-encoded porin loss or efflux pump overexpression may form the basis of resistance in these strains.

Monitoring patient-to-patient spread in an intensive care unit. Fifty-four (18.8%) 3GC-R isolates obtained during this study originated from ICU-admitted patients. Resistance to ceftriaxone and cefoxitin was identified in 100% and 31.4% of isolates, respectively, and meropenem resistance was identified in three (5.5%) isolates. Due to their dominance, only *E. coli* and *K. pneumoniae* genomes were analyzed to assess for potential transmission events. Among the *E. coli* population (Figure 2), isolates SCHI0016.S.60 and SCHI0016.S.62, and SCHI0016.S.265 and SCHI0016.S.46, were separated by 10 SNPs and 2 SNPs, respectively; however, both pairs came from individual patients, reflecting expected levels of within-host evolution of a single infecting strain. *E. coli* isolates SCHI0016.S.42 and SCHI0016.S.294 were very similar, being separated by 13 SNPs. Although both strains were isolated from ICU admitted patients, SCHI0016.S.294 was isolated 306 days after

SCHI0016.S.42 with no other linking epidemiology identified. Additionally, no similar strains were isolated from ICU patients over this time period suggesting that these do not represent transmission within the ICU. *E. coli* isolates SCHI0016.S.172 and SCHI0016.S.274 differed by approximately 300 SNPs, with no epidemiological or temporal correlation between patients. A similar number of SNPs separated isolates SCHI0016.S.112 and SCHI0016.S.229, again with no demonstrable epidemiological link. Among the *K. pneumoniae* ICU strains (Figure 3), the closest isolates, SCHI0016.S.15 and SCHI0016.S.261, differed by 361 SNPs, and again had no epidemiological link. All patients in our ICU, and all patients in other hospital wards that are infected or colonized with confirmed ESBL-producing *E. coli* and *K. pneumoniae*, have a single room with an assigned nurse (1:1 ratio), with contact precautions exercised as standard practice. In additional, hand hygiene rates in our hospital ICU are generally above 80% (Australian national benchmark) [37]. Taken together, our results demonstrate no evidence of ESBL transmission in the ICU ward over the study period, suggesting the implementation of effective hygiene measures by ICU staff.

Population structure of E. coli and K. pneumoniae genomes. We subjected an additional 36 strains to WGS (total *n*=98) to capture a snapshot of genomic diversity and population structure in our samples (Figures 2 and 3). *In silico* MLST of the 79 genome-sequenced *E. coli* strains found that ST131 was most common ST (*n*=11; 13.9%). This finding is consistent with a recent study of the population structure of Australian, New Zealand, and Singaporean multidrug-resistant *E. coli* isolates, where ST131 was identified as the dominant strain [10]. The highly successful ST131 clone has spread worldwide, where it causes urinary tract and bloodstream infections, particularly community-associated infections [38]. Overall, depending on the country, ST131 accounts for 12–27% of all community-acquired *E. coli* infections [39]. In addition, transmission events have been documented among hospitalized patients, although person-to-person spread appears to be more important between

366 household contacts [40]. Among our ST131 isolates, we found high frequencies of
367 fluoroquinolone resistance-conferring variants ($GyrA_{S83L}$, $GyrA_{D87N}$, and $ParC_{S80I}$) alongside
368 frequent carriage of plasmid-borne CTX-M-14 (CTX-M-9 group) and CTX-M-15 (CTX-M-9
369 group) ESBLs. We also identified three strains belonging to the emerging multi-drug resistant
370 pandemic clone, ST1193: SCHI0016.S.133, SCHI0016.S.172 and SCHI0016.S.274, although the
371 latter strain was excluded from the phylogenetic analysis due to the presence of mixtures.
372 These isolates also possessed the same three fluoroquinolone-resistant SNPs as the ST131
373 strains. It has been proposed that ST1193 strains evolved fluoroquinolone resistance
374 relatively recently through multiple recombination events with other AMR *E. coli* strains
375 [41].
376
377 Phylogenomic analysis of our 10 *K. pneumoniae* genomes identified several distinct lineages,
378 which comprised two ST14 strains and one each of ST17, ST307, ST309, ST395, ST656, ST697,
379 ST873, and ST969. Of note, the hypervirulent and globally disseminated ST258 clone, which
380 is responsible for a large number of hospital deaths [42], was not identified in our dataset.
381 However, we identified another globally disseminated clone, ST395, which has also been
382 retrieved from clinical cases in Europe and Asia [43]. Concerningly, this ST395 isolate,
383 SCHI0016.S.137, carried both the ESBL genes bla_{SHV-11} and $bla_{CTX-M-15}$ and the OXA-48-like
384 carbapenemase, $bla_{OXA-232}$, the latter of which confers low-level meropenem resistance.
385
386 This study had several recognized limitations. First, isolates were only collected from a
387 hospital setting rather than from both nosocomial and community sources. It is well-
388 established that differences in AMR patterns and their associated molecular mechanisms
389 exist between hospital and community settings. Second, the absence of certain high-risk
390 populations within our hospital (e.g. solid organ or hematopoietic stem cell transplantation,
391 burns) limits the generalizability of our findings to other settings. Third, our PCR assays can

only identify the presence or change in copy number of the most common ESBL and *ampC* genes. Although these loci make up the majority (>90%) of β-lactamase enzymes present in 3GC-R Enterobacteriaceae, they do not capture all potential ESBLs, leaving less common ESBL and *ampC* genes unidentified. In addition, PCR assays for bla_{TEM} or bla_{SHV} cannot discriminate between ESBLs and non-ESBLs, with additional assays or concurrent WGS required to confirm ESBL status. Our assays also cannot detect chromosomal changes leading to AMR (e.g. porin mutations or efflux pump up-regulation), making these assays less useful for 3GC-R bacteria that frequently encode these AMR determinants (e.g. *Pseudomonas aeruginosa*). One way to address detection issues is to undertake WGS on all samples; however, as demonstrated in this study, some AMR determinants will likely remain cryptic due to their novelty, thereby requiring additional functional work to verify their causative nature. Fourth, our PCR was unable to distinguish whether β-lactamase genes were present on low-copy plasmids or were chromosomally incorporated, which has implications for AMR transmissibility between and among bacterial populations. Finally, ~40% of samples were obtained from rectal swabs, which have inherent limitations in detection of multidrug-resistant organisms due to the high microbial burden from this specimen type coupled with difficulties in adequate sample collection. It is therefore likely that we did not capture all 3GC-R clinical isolates during our study period due to the inherent difficulties associated with this sampling technique, and thus may have missed potential transmission events in the hospital.

Conclusion

Taken together, our findings identified a predominance of CTX-M (and to a lesser extent, CMY) β-lactamases among 3GC-R Enterobacteriaceae in the Sunshine Coast region. These findings are consistent with global findings of Gram-negative bacterial AMR determinants, particularly among *E. coli*. Our study demonstrates the likely absence of 3GC-R Enterobacteriaceae transmission over a one-year period in an ICU, indicating effective infection control processes for these pathogens. We show that rapid molecular assays, such as our multiplex PCR, can detect common β-lactamase genes in a clinical setting within 40 minutes, including directly from bacterial culture. Further research is required to determine whether our molecular assays can be used directly on clinical specimens, and whether the implementation of molecular assays and more advanced technologies (e.g. WGS) is associated with improved patient outcomes in the hospital setting.

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Figures

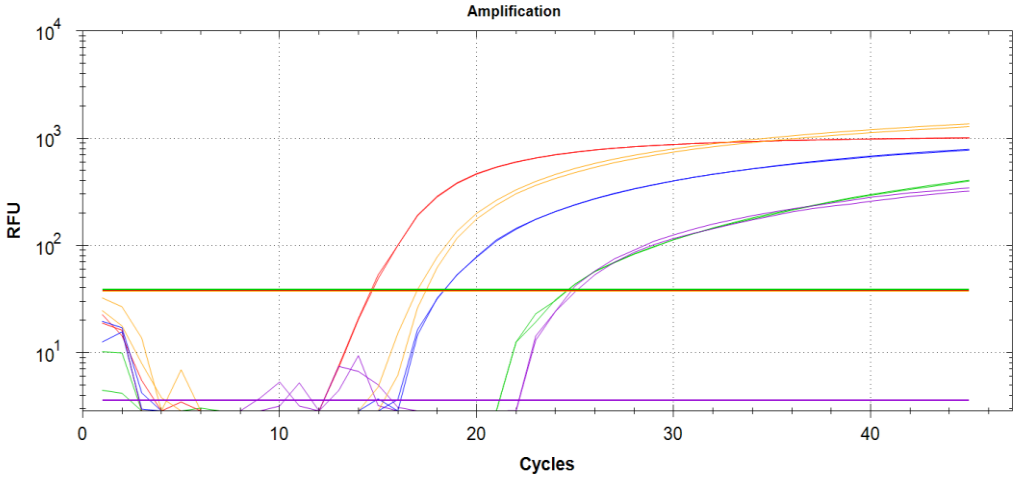


Figure 1: Pentaplex PCR performance on third-generation cephalosporin-resistant Enterobacteriaceae. Red, 16S rDNA gene; orange, CTX-M group 9; blue, TEM; green, CTX-M group 1; purple, SHV.

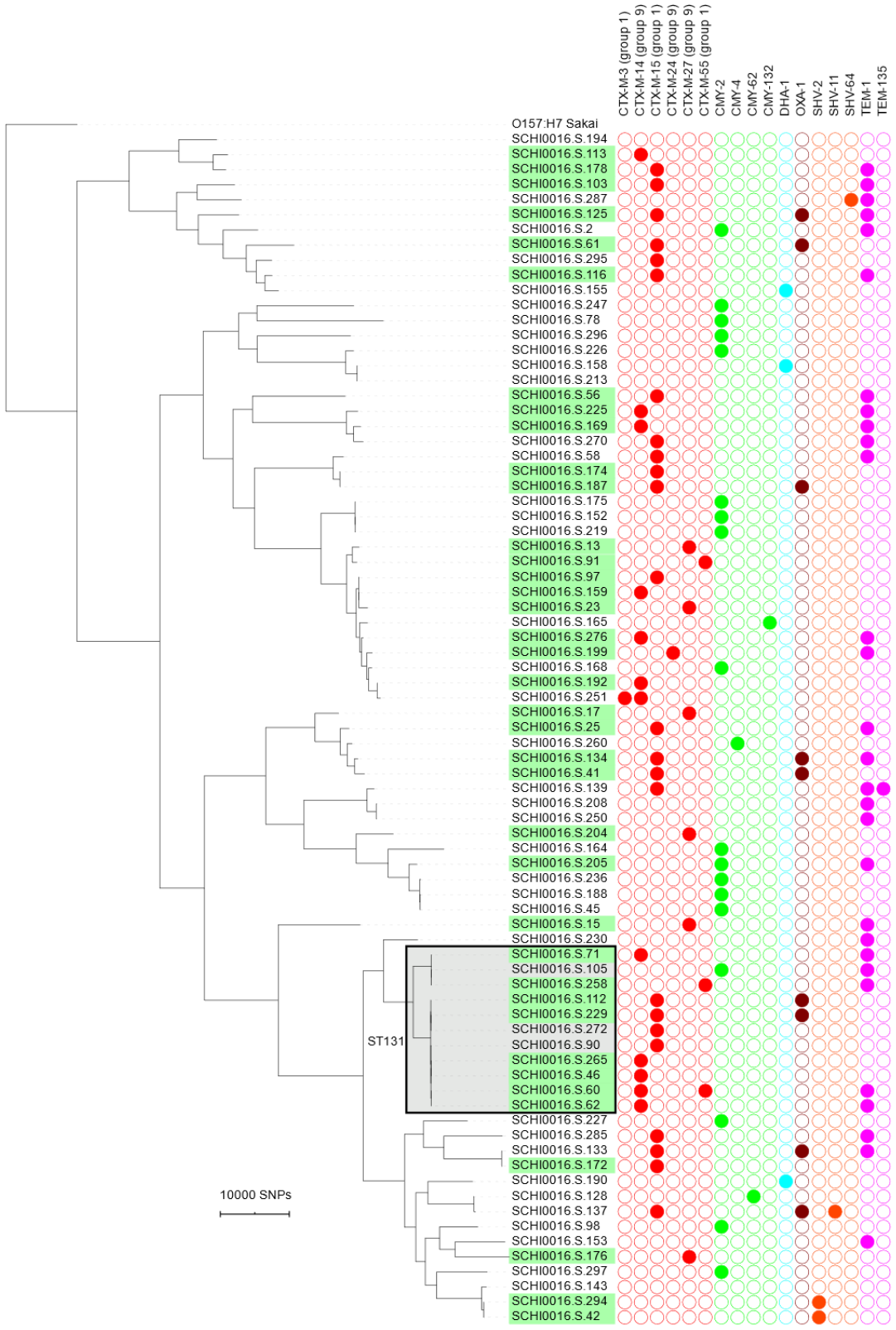
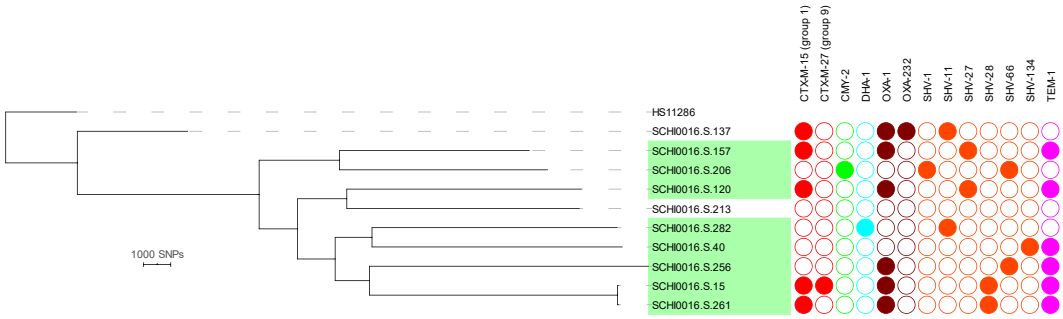


Figure 2. Whole-genome maximum parsimony phylogeny of 79 third-generation cephalosporin-resistant *Escherichia coli* isolates and their β-lactamase gene complement. Sequence type (ST)131 is indicated by the gray-shaded box; strains isolated from intensive care unit patients are highlighted in green.



443

444 **Figure 3. Whole-genome phylogeny and presence of β-lactamase enzymes in the *Klebsiella***

445 ***pneumoniae strains* isolated in our study.** Isolates shaded in green were isolated from the

446 intensive care unit.

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Tables

Table 1: Summary of antimicrobial resistance mechanisms among 288 clinical Enterobacteriaceae isolates retrieved from 258 patients admitted to a Southeast Queensland hospital over a 14-month period.

Organism	***E. coli* (*n*=231)**	***K. pneumoniae* (*n*=49)**	***K. aerogenes (n=1)***	***K. oxytoca* (*n*=3)**	***E. cloacae* (*n*=4)**
Phenotypic antimicrobial resistance[a]					
Ceftriaxone	223 (96.5%)	46 (93.9%)	1 (100%)	3 (100%)	4 (100%)
Ceftazidime	215 (93.1%)	47 (95.9%)	1 (100%)	2 (66.7%)	4 (100%)
Cefepime	201 (87.0%)	43 (87.8%)	1 (100%)	2 (66.7%)	3 (75.0%)
Cefoxitin	64 (27.7%)	12 (24.5%)	1 (100%)	0 (0%)	4 (100%)
Piperacillin-tazobactam	65 (28.1%)	31 (63.3%)	1 (100%)	3 (100%)	4 (100%)
Ticarcillin-clavulanate	186 (80.5%)	46 (93. 9%)	0 (0%)	3 (100%)	3 (75.0%)
Meropenem	3 (1.3%)	2 (4.1%)	0 (0%)	0 (0%)	3 (75.0%)
ESBL phenotypic test[b]					
Positive	204 (88.3%)	46 (93.9%)	0 (0%)	2 (66.7%)	4 (100%)
Negative	27 (11.7%)	3 (6.1%)	1 (100%)	1 (33.3%)	0 (0%)
ESBL and *ampC* gene prevalence					
$bla_{\text{CTX-M-1}}$	104 (45.0%)	36 (73.5%)	0 (0%)	2 (66.7%)	2 (50.0%)
$bla_{\text{CTX-M-9}}$	89 (38.5%)	4 (8.2%)	0 (0%)	0 (0%)	0 (0%)
bla_{SHV}	9 (3.9%)	38 (77.6%)	0 (0%)	0 (0%)	1 (25.0%)
bla_{TEM}	93 (40.3%)	33 (67.3%)	0 (0%)	2 (66.7%)	2 (50.0%)
bla_{CMY}	30 (13.0%)	3 (6.1%)	0 (0%)	0 (0%)	1 (25.0%)
PCR-negative[c]	7 (3.0%)	1 (4%)	1 (100%)	1 (33.3%)	1 (25.0%)

[a]Antimicrobial susceptibility testing performed by automated method (VITEK 2®) and EUCAST breakpoints applied

[b]ESBL phenotypic testing performed by disc diffusion method

[c]DNA integrity was confirmed by 16S rDNA PCR [15], which forms part of the pentaplex assay. Three PCR negative *E. coli* strains encoded $bla_{\text{DHA-1}}$, one *E. cloacae* encoded $bla_{\text{ACT-17}}$, one *K. oxytoca* encoded a $bla_{\text{OXY-2-1}}$, a second *K. oxytoca* (included as a mixture with *K. pneumoniae*) encoded $bla_{\text{OXY-2-4}}$, and 5/11 isolates had no β-lactamases identified despite whole-genome sequencing.