Wastewater-based monitoring and genomic characterisation of antibiotic-resistant bacteria in the Sydney community

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Wastewater-based monitoring and genomic characterisation of antibiotic-resistant bacteria in the Sydney community

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Abstract

Current healthcare infection surveillance rarely monitors the distribution of antimicrobial resistance (AMR) in bacteria beyond clinical settings in Australia and overseas. This results in a significant gap in our ability to fully understand and manage the spread of AMR in the general community. This thesis explores whether wastewater-based monitoring could reveal geospatial-temporal and demographic trends of antibiotic-resistant bacteria in the urban area of Greater Sydney, Australia. Untreated wastewater from 25 wastewater treatment plants sampled between 2017 and 2019 consistently contained extended-spectrum β-lactamases-producing *Enterobacteriaceae* (ESBL-E) isolates, suggesting its endemicity in the community. Carbapenem-resistant *Enterobacteriaceae* (CRE), vancomycin-resistant enterococci (VRE), and methicillin-resistant *Staphylococcus aureus* (MRSA) isolates were occasionally detected. Demographic and healthcare infection-related factors correlated with the ESBL-E load, and demographic variables influenced the VRE load. In contrast, the healthcare infection-related factor mainly drove the CRE load. These findings demonstrate the potential of wastewater-based surveillance to understand the factors driving AMR distribution in the community.

The subsequent thesis work covers the genomic characterisation of selected ESBL-E and CRE wastewater isolates to reveal their nature, origin, and underlying resistance mechanisms. Phylogenetic analysis showed that *Escherichia coli* isolates were related to high-risk human-associated pandemic clones and non-human-associated clones. The *Klebsiella pneumoniae* and *K. variicola* isolates were related to globally disseminated and emerging human-associated clones, and some were detected for the first time in Australia. Genomic analysis also indicated novel resistance mechanisms against nitrofurantoin in *E. coli*, and against piperacillin/tazobactam and ticarcillin/clavulanic acid in *Klebsiella* isolates. The virulence gene content indicated that some *E. coli* and *Klebsiella* isolates were likely associated with infections, while the asymptomatic carriage was suggested for other isolates. These results demonstrate a clear potential for wastewater-based surveillance to monitor the emergence and dissemination of resistance in non-clinical isolates, and in particular, isolates from the community and non-human sources.

The findings of this study can complement healthcare infection surveillance to inform management strategies to mitigate the emergence and dissemination of AMR and important human pathogens in the general community.

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List of Abbreviations

aa	Amino acid
AAC	Aminoglycoside acetyltransferases
AES	Advanced expert system
AMR	Antimicrobial resistance
AMU	Antimicrobial use
ANT	Aminoglycoside nucleotidyltransferase
APEC	Avian pathogenic *Escherichia coli*
APH	Aminoglycoside phosphotransferase
AST	Antibiotic susceptibility testing
AURA	Antimicrobial Use and Resistance in Australia
BIGSdb	Bacterial isolate genome sequence database
CA	Community-acquired
CAI	Community-acquired infection
CDS	Coding sequence
CFU	Colony-forming unit
CG	Clonal group
Clb	Colibactin
CLSI	Clinical and Laboratory Standards Institute
CMY	Cephamycinase
Col	Colicinogenic
CR	Carbapenem-resistant
CRE	Carbapenem-resistant *Enterobacteriaceae*
CTX-M	Cefotaximase-Munich
del	Deletion
EAEC	Enteroaggregative *Escherichia coli*
EC	*Escherichia coli*
Ent	Enterobactin
ESBL	Extended-spectrum β-lactamases
ESBL-E	Extended-spectrum β-lactamases-producing *Enterobacteriaceae*
FNR	Flow normalised relative
GAP-AMR	Global Action Plan on Antimicrobial Resistance
GES	Guiana extended-spectrum
GLASS	Global Antimicrobial Resistance Surveillance System
GP	General practitioner
GTR	General time reversible
HAI	Healthcare-acquired infection
HBA	Horse blood agar
HGT	Horizontal gene transfer
IMP	Imipenemase
INDEL	Insertion-deletion
INS	Insertion
Iro	Salmochelin
Iuc	Aerobactin
kbp	Kilobase pairs
KP	*Klebsiella pneumoniae*
KPC	*Klebsiella pneumoniae* carbapenemases

KV	*Klebsiella variicola*
LB	Luria-Bertani
LOD	Limit of detection
LPS	Lipopolysaccharide
MALDI-TOF MS	Matrix-assisted laser desorption ionisation-time of flight mass spectrometry
MB	Mesh block
MBL	Metallo-β-lactamases
Mbp	Megabase pairs
MDR	Multidrug-resistant
MGE	Mobile genetic element
MIC	Minimum Inhibitory Concentration
ml	Millilitre
MLST	Multilocus sequence typing
MRSA	Methicillin-resistant *Staphylococcus aureus*
NDM	New Delhi metallo-β-lactamase
nt	Nucleotide
OMP	Outer membrane porin
OXA	Oxacillinase
PMQR	Plasmid-mediated quinolone resistance
PROVEAN	Protein variation effect analyser
PVL	Panton-Valentine leukocidin toxin
QRDR	Quinolone resistance-determining region
RACF	Residential aged care facility
SA1	Statistical area 1
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SHV	Sulfhydryl variable
SNP	Single nucleotide polymorphism
ST	Sequence type
TEM	Temoniera
UPEC	Uropathogenic *Escherichia coli*
UTI	Urinary tract infection
VIF	Variance inflation factor
VIM	Verona integron-encoded metallo-β-lactamase
VRE	Vancomycin-resistant enterococci
WBE	Wastewater-based epidemiology
WGS	Whole-genome sequencing
WWTP	Wastewater treatment plant
XDR	Extensively drug-resistant
Ybt	Yersiniabactin

Chapter 1 General Introduction

1.1 Background

It is difficult to overstate the beneficial impact that the control of pathogens has had on our standard of living. Management and control of microbial infections are well documented in ancient China, Egypt, Greece, and other parts of the world (Sengupta et al., 2013). In the initial era of medicine, effective control and management of infections began with the discovery of penicillin by Sir Alexander Fleming in 1928 (Fleming, 1929). More than 150 other types of antibiotics have been discovered in the decades that followed. These antibiotics not only reduced the morbidity and mortality attributable to infectious diseases with antibiotics but also permitted ground-breaking advances in medicine and surgery, such as organ transplantation (Gould & Bal, 2013).

The use of these drugs coincided with the rapid emergence of resistant bacterial strains (Davies & Davies, 2010), that for some resistances were recognised soon after antibiotics were discovered (Abraham & Chain, 1988). Eventually, pathogens developed resistance against almost all antibiotics that have been developed so far (Falagas & Bliziotis, 2007; Souli et al., 2008; Karakonstantis et al., 2020). Currently, the emergence and spread of multidrug-resistant (MDR) bacterial pathogens have become a significant public health threat due to the difficulty in treatment and the expense associated with the development of new antibiotics (WHO, 2014; Silver, 2011). Most often, few or no effective antimicrobial agents remain to treat these MDR bacterial infections (Jacoby & Munoz-Price, 2005; Arias & Murray, 2009; David & Daum, 2010; Arias & Murray, 2012; Patrice Nordmann et al., 2012). In 2013, the Centers for Disease Control and Prevention (CDC) declared that a 'post-antibiotic era' was evident, and by 2014 the World Health Organization (WHO) described antimicrobial resistance (AMR) as one of the significant global health issues (Michael et al., 2014; WHO, 2014). In the United States, approximately 2.8 million people are affected by severe antibiotic-resistant infections each year, and 35,000 deaths have occurred as a direct result (CDC, 2019). In 2019, approximately 1.27 million deaths worldwide were directly related to AMR (Murray et al., 2022).

1.2 Mechanisms of antibiotic resistance and its acquisition

Bacterial resistance to antibiotics can be intrinsic (inherent, natural, and chromosomal) or acquired (alteration of the bacterial genome). Intrinsic resistance is the natural ability of an organism to resist an antibiotic due to its inherent structural and functional characteristics (Cox & Wright, 2013). This type of resistance is found in various bacterial species, is independent of an antibiotic's selection pressure, and is not attributed to horizontal gene transfer. For example, intrinsic resistance to ampicillin was observed in *Klebsiella* species due to the production of β-lactamases enzyme that destroys the antibiotic before it reaches its target: penicillin-binding proteins (PBP) (Bouza & Cercenado, 2002). *Enterobacter* species constitutively produce AmpC β-lactamase, that can resist the action of ampicillin, amoxicillin, amoxicillin/clavulanic acid, first-generation cephalosporins, and cefoxitin (Bouza & Cercenado, 2002).

Acquired resistance results from alteration of the bacterial genome either by mutations (Martinez & Baquero, 2000) or by horizontal gene transfer (HGT) (Ochman et al., 2000; Baquero et al., 2002; Normark & Normark, 2002; Baquero & Coque, 2011). In bacteria, genetic mutations conferring resistance can occur frequently, but without considerable amplification, this has only minor clinical significance (Martinez & Baquero, 2000; Davies & Davies, 2010). Horizontal acquisition of external genetic material in bacteria occurs through three main mechanisms, transformation, transduction, and conjugation (Davison, 1999; Norman et al., 2009; von Wintersdorff et al., 2016). Acquired resistance is of greater concern as it allows initially susceptible bacteria to rapidly develop resistance against an antibiotic that enables the resistant bacteria to proliferate and spread under the selective pressure of that drug. Several mechanisms confer resistance for each class of antibiotics that depends on the bacterial species and its genetic makeup. The main mechanisms of resistance include (i) enzymatic inactivation or modification of the antibiotic (e.g. β-lactamases and aminoglycoside-modifying enzymes), (ii) decreased antibiotic uptake (e.g. mutated porins), (iii) increased efflux (e.g. upregulated efflux pumps), (iv) inactivation or modification of the antibiotic target (e.g. methylation or mutation of ribosomal RNA in resistance to erythromycin), (v) introduction of a new antibiotic-resistant target (e.g. horizontal acquisition of the *mecA* gene encoded for a β-lactam-insensitive penicillin-binding protein, PBP2a, in resistance to methicillin) or other molecular bypass mechanisms (e.g. a drug-resistant variant of the chromosomal target

enzyme dihydrofolate reductase that confers resistance to trimethoprim) (Weisblum, 1995; Huovinen, 2001; Jacoby & Munoz-Price, 2005; Piddock, 2006; Chambers & Deleo, 2009; Ramirez & Tolmasky, 2010; Wozniak et al., 2012).

1.3 Emergence and spread of antibiotic resistance in healthcare settings

Healthcare-acquired infections (HAIs) are defined as infections acquired by patients during care that are not present or incubating at the time of admission (www.cdc.gov.hai/index.html). Any infection that develops 48 hours or longer after admission to a healthcare facility is considered an HAI (Ducel et al., 2002). There are many factors that promote the development and transmission of infections within a healthcare facility, most of which are related to poor infection control practices. These factors include poor hand hygiene of staff before contact with patients, overcrowded wards requiring high-touch environmental cleaning, presence of immunocompromised patients, long duration of invasive devices such as central catheters, and poor equipment sterilisation (Ducel et al., 2002). Despite requiring a general HAI safety practice as mentioned above, the prevention of HAI will prevent AMR in patients in developed and resource-poor countries. HAIs are a major problem in both developed and developing countries, except that the prevalence of HAIs in developing countries is generally unknown but estimates at 15.5% (Allegranzi et al., 2011). In developed healthcare facilities, 3.2% and 6.5% rates of HAI have been observed in the United States and European countries, respectively (Magill et al., 2018; Suetens et al., 2018). In Australia, there have been approximately 165,000 HAIs per year (Mitchell et al., 2017).

HAIs are associated with increased morbidity, mortality, and healthcare-related costs (Mulvey & Simor, 2009). The emergence of antibiotic resistance in healthcare facilities is a major health threat (Michael & Simor, 2009). AMR not only contributes to patient illness and death but also increases treatment complexity and length of hospital stays, and places a substantial burden on individual patients, the health system, and health service organisations (Hunter & Reeves, 2002; Frimodt-Møller et al., 2007; Smith & Coast, 2012).

In healthcare settings, bacterial resistance is generally attributed to selective pressures created by excessive and inappropriate use of antibiotics (WHO, 2001; Struelens, 1998; Weinstein, 2001). In the United States, 258.2 million courses of antibiotics were prescribed (793 prescriptions per 1,000 people) in 2017, with penicillins and macrolides being the most frequently prescribed (CDC, 2017). Antimicrobial use is also high in Australia compared to other countries, with an estimated 22.7 defined daily doses (DDD) per 1,000 inhabitants per day in 2017-2018 (ECDC, 2019; AURA, 2019). According to the Pharmaceutical Benefits Scheme (PBS) and the Repatriation Pharmaceutical Benefits Scheme (RPBS), the most commonly dispensed antimicrobial groups in 2017 were β-lactams, followed by tetracyclines and macrolides (AURA, 2019). Inappropriate prescribing and empirical use of antibiotics due to shortcomings in rapid and accurate diagnosis of infections results in excessive and improper use of clinically important antibiotics (Michael et al., 2014). Inappropriate prescribing is defined as the administration of antibiotics for conditions that did not require an antibacterial, for example, to treat viral or upper respiratory tract infections and the prescribing of unnecessary broad-spectrum antibiotics and the incorrect dose (i.e. longer than necessary) and routes (intravenous instead of oral) (Maxwell, 2016; Spivak et al., 2016). According to the CDC, at least 30% of antibiotics used in emergency departments and doctors' practices in the United States were unnecessarily prescribed (CDC, 2019). According to the AURA surveillance system in Australia in 2017, 23.5% of the prescriptions were deemed inappropriate, and 32.7% did not comply with the guidelines (AURA, 2019). The extensive and inappropriate use of antibiotics kills or inhibits the majority of susceptible organisms and provides an environment where resistant organisms can proliferate and become predominant (McGowan Jr, 1983; Cosgrove & Carmeli, 2003; Mulvey & Simor, 2009).

Among the most challenging MDR organisms of clinical importance are extended-spectrum β-lactamases-producing *Enterobacteriaceae* (ESBL-E) (Jaoby & Munoz-Price, 2005), carbapenem-resistant *Enterobacteriaceae* (CRE) (Nordmann et al., 2012), vancomycin-resistant enterococci (VRE) (Arias & Murray, 2012), and methicillin-resistant *Staphylococcus aureus* (MRSA) (David & Daum, 2010). An overview of the epidemiology and distribution of these critically important pathogens in healthcare settings is provided in the following section.

1.3.1 Epidemiology and distribution of ESBL-E in healthcare settings

ESBL-E poses unique challenges to treatment. ESBLs are enzymes capable of hydrolysing most β-lactam antibiotics, including third-generation cephalosporins (i.e. cefotaxime, ceftriaxone, and ceftazidime) and monobactams (i.e. aztreonam), but not cephamycins (i.e. cefotetan and cefoxitin) and carbapenems (i.e. meropenem, imipenem, and ertapenem) (Bradford, 2001). ESBL-producing organisms also have the ability to acquire resistance to other classes of antibiotics, such as aminoglycosides, trimethoprim, quinolones, tetracyclines, and cotrimoxazole, which further reduce therapeutic options (Lautenbach et al., 2001; Pitout et al., 2005; Schwaber et al., 2005; Morosini et al., 2006). ESBLs are most commonly found in *Klebsiella pneumoniae* and *Escherichia coli* but have been less frequently reported in *Citrobacter, Enterobacter, Proteus, Salmonella, Serratia,* and other genera within the *Enterobacteriaceae* family (Thomson & Moland, 2000). The most common infections associated with ESBL-E in healthcare settings include respiratory tract, wound, bloodstream, urinary tract infections (UTIs), intra-abdominal infections and septicaemia (Pitout & Laupland, 2008). Patient-to-patient transmission of ESBL-E can occur through contaminated hands of healthcare workers, colonisation of the inanimate environment, and medical equipment (Paterson & Bonomo, 2005).

ESBL enzymes were first recognised in clinical isolates in the early 1980s, which were found to originate from point mutations in the parent enzymes, such as temoniera (TEM)- or sulfhydryl variable (SHV)-types β-lactamases (Jacoby & Munoz-Price, 2005; Dhillon & Clark, 2012). However, a shift in the genotypic makeup of ESBLs became evident at the beginning of the 21st century (Bonnet, 2004; Falagas & Karageorgopoulos, 2009). For example, in *E. coli* and *K. pneumoniae*, the plasmid-mediated cefotaximase-Munich (CTX-M) genotype has become more prevalent, which originated from the chromosomally encoded enzyme of environmental *Kluyvera* species (Bonnet, 2004; Pitout et al., 2005; Falagas & Karageorgopoulos, 2009). The new CTX-M enzymes appear to have an improved capacity to cause outbreaks, since the genes encoding the enzyme have been mobilised into conjugative plasmids that can be readily transferred between pathogenic bacteria (Bonnet, 2004; Cantón & Coque, 2006). To date, more than 50 variants of CTX-M enzymes associated with outbreaks have been identified

(Livermore & Hawkey, 2005; Falagas & Karageorgopoulos, 2009). Other clinically relevant ESBLs mainly include OXA-, GES-, TLA-, VEB-, PER-, BES-, BEL-, IBC-, and SFO-types (Jacoby et al., 2005; Livermore & Hawkey, 2005).

In acute care hospitals, an increased prevalence of ESBL-E has been observed (Doi et al., 2017; Spadafino et al., 2014). In the United States, the rates of ESBL producers in uropathogenic *E. coli* have doubled from 7.8% in 2010 to 15.7% in 2017 (Lob et al., 2016; Critchley et al., 2019). Between 2018 and 2019, 11.2% of *K. pneumoniae* strains isolated from the United States hospitals were ESBL producers and, unlike *E. coli* isolates, the majority were CTX-M-types (Karlowsky et al., 2022). In Europe, different detection rates of ESBL producers were reported for both *E. coli* (14.8-27.7%) and *K. pneumoniae* (20.7-31.4%) in 2018-2019 (Karlowsky et al., 2022). In Canada, the ESBL producer rates have increased for both *E. coli* (from 3.3% in 2007 to 11.2% in 2018) and *K. pneumoniae* (from 1.3% in 2007 to 9.3% in 2018) (Karlowsky et al., 2021). In Southeast and East Asia, the detection rates of ESBL-producing *E. coli* (ESBL-EC) isolates in hospitals were 20-42.2%, with an increasing trend observed in many countries (Jean et al., 2016, Karlowsky et al., 2022). In Australia, ESBL producers were detected in approximately 22.2% of *E. coli* isolates and 7.6% of *Klebsiella* species from HAIs in 2019 (AGAR, 2019).

Undoubtedly, carbapenems are the first-line treatment of choice against ESBL-E infections (Harriset al., 2015). However, as ESBL-E infections have been increasingly detected worldwide, the increased use of carbapenems (last resort drugs) has become an inevitable choice (Harris et al., 2015). The resultant overuse of carbapenems is concerning due to the potential to select CRE (Schwaber & Carmeli, 2008).

1.3.2 Epidemiology and distribution of CRE in healthcare settings

The emergence and spread of CRE that exhibit resistance against almost all antibiotics, including last resort antibiotics such as carbapenems, cause increased therapeutic hurdles for patients in acute and long-term care facilities (Tzouvelekis et al., 2012; Ventola, 2015). Carbapenem resistance is usually caused by the production of carbapenemase enzymes capable of hydrolysing carbapenems (Queenan & Bush, 2007). In *Enterobacteriaceae*, carbapenem resistance is also attributed to the deletion or reduction of outer membrane porin (OMP) in the presence/absence of production of ESBLs and/or

AmpC β-lactamases (Nordmann et al., 2012). Infections often associated with CRE include pneumonia, UTIs, intra-abdominal and device-associated infections, especially in immunocompromised patients (Peleg et al., 2005; Leavitt et al., 2007; Akova et al., 2012; Tzouvelekis et al., 2012). Infections with CRE prolong hospital stays and cause increased mortality (18-48%) (Akova et al., 2012). Exposure to healthcare and long-term care, prior antibiotic use, and the presence of invasive catheters and drains are considered important risk factors for CRE colonisation (Bart et al., 2015; Bhargava et al., 2014; Dhar et al., 2016; Teo et al., 2012). Treatment options for serious CRE are extremely limited. Combinations of carbapenems, colistin, tigecycline, fosfomycin, polymyxins, tigecycline, aminoglycosides, ceftazidime-avibactam, and meropenem-vaborbactam are often used (Falagas & Kasiakou, 2005; Falagas & Kopterides, 2007; Falagas et al., 2014; Oliva et al., 2014). However, the evidence supporting their clinical use is limited, and their use is often limited due to toxicities or other pharmacokinetic shortcomings (Garonzik et al., 2011; van Duin et al., 2013; Morrill et al., 2015; Rigatto et al., 2016).

Worldwide, CRE organisms are rare. However, over the past decade, untreatable or difficult-to-treat infections by CRE bacteria have increased in healthcare settings, and the CDC recognised CRE as an urgent threat to public health (Jacob et al., 2013; CDC, 2019). According to the CDC, more than 13,100 HAIs in the United States are caused each year by CRE, and approximately 1,100 deaths are caused by the two most common CRE, such as *E. coli* and *Klebsiella* species (CDC, 2019). In the United States, South America, Greece, Israel and China, reports of plasmid-mediated *K. pneumoniae* carbapenemases (KPC) have been increasing (Nordmannm et al., 2009). KPC is the predominant carbapenemase in the United States, which was first identified in a *K. pneumoniae* strain in 1996 and other less common carbapenemases, including New Delhi metallo-β-lactamase (NDM)-, Verona integron-encoded metallo-β-lactamase (VIM)-, and oxacillinase-48 (OXA-48)-types (Guh et al., 2014). Countries may have different CRE epidemiology, for example, in Israel, KPC is the most common carbapenemase, and in Japan, imipenemase (IMP) is endemic, while VIM is reported to be endemic in Greece (Cantón et al., 2012). In India, NDM-1 emerged, and OXA-48-type carbapenemases originated in Turkey and became endemic; however, these are encoded on transmissible elements enabling global dissemination (Yong et al., 2009; Carrer et al., 2010; Kumarasamy et al., 2010; Moellering Jr, 2010; Nordmann et al., 2011; Sengupta et al.,

2013). In Australia, a CRE outbreak was first documented in 2012, where seven cases were identified with a mortality rate of 40% (Chang et al., 2015). Although IMP-type carbapenemase is endemic on the Australian east coast, CRE is still rare, with less than 0.1% in *E. coli* and 0.6% *Klebsiella* species resistant to carbapenems (meropenem) in 2019, based on isolates from blood and urine cultures (AURA, 2021). There is thus substantial variability in the distribution of carbapenemases on continents, nationally and regionally (van Duin & Doi, 2017). Therefore, awareness of the factors that contribute to the emergence and spread of CRE is essential to prevent further spread.

1.3.3 Epidemiology and distribution of VRE in healthcare settings

VRE is mostly restricted to healthcare settings and causes UTIs, bloodstream, and surgical site infections, mainly in vulnerable individuals such as elderly or immunocompromised patients (Sengupta et al., 2013; CDC, 2019; AURA, 2021). The major concern associated with enterococcal species is their tolerance to heat, some alcohol preparations, and chlorine (Bradley & Fraise, 1996), making their elimination difficult and enabling dissemination in the hospital environment. Resistance to vancomycin is usually attributed to the acquisition of plasmid-associated genes, for example, the *vanA* or *vanB* genes (Leclercq & Courvalin, 1997). These genes encode the synthesis of modified cell wall precursors that do not bind glycopeptides (i.e. vancomycin). In addition, these genes are often associated with plasmids that allow them to spread from enterococci to MRSA, further complicating the treatment of these infections (Appelbaum, 2006). A Canadian surveillance program reported that 15% of inpatients with VRE were also colonised with MRSA (Ofner-Agostini et al., 2008). The prevalence of VRE remains relatively low worldwide, except in the United States and some European countries (Arias & Murray, 2012; CDC, 2019; ECDC, 2019; Shrestha et al., 2021). Worldwide, high levels of ampicillin resistance have been reported in *E. faecium* during the past 20 years, including in Australia, which increases the use of vancomycin for treatment (AURA, 2021). The inherent β-lactam resistance in enterococci causes significant treatment difficulties when it develops vancomycin resistance; this occurs more frequently in *E. faecium* than in *E. faecalis* (Fisher & Phillips, 2009). In Australia, the rate of vancomycin resistance in clinical *E. faecalis* was very low (< 1%) in 2018-2019 compared to resistance rates in *E. faecium* (> 40%) (AURA, 2021).

Infections with VRE require treatment with reserved antimicrobial agents, such as teicoplanin or daptomycin.

1.3.4 Epidemiology and distribution of MRSA in healthcare settings

S. aureus is another common cause of HAI, such as intravascular line infections with bacteraemia, surgical site infections, and infections of prosthetic devices (Spelman, 2002; McLaws & Taylor, 2003). Bacteraemia associated with *S. aureus* infections has a mortality rate of 15–30% (van Hal et al., 2012). MRSA infection represents one of the leading causes of HAI that are generally associated with significant morbidity, mortality, duration of stay, and cost burden (Cosgrove et al., 2005; Lodise & McKinnon, 2007; Turnidge et al., 2016). MRSA bacteraemia is usually associated with patients in the intensive care unit with central line insertions. MRSA was first detected in 1960 (Jevons, 1961), shortly after introducing methicillin (second-generation β-lactam antibiotic) into clinical practice. In the late 1970s, MRSA emerged as a major pathogen in healthcare settings worldwide (Brumfitt & Hamilton-Miller, 1989; Peacock et al., 1990; Simor et al., 2001). MRSA infection is now endemic in hospitals around the world (Turnidge et al., 2016; Lee et al., 2018; Turner et al., 2019). Currently, MRSA has been detected in up to 30–50% of invasive isolates from hospitals (Dantes et al., 2013; Lee et al., 2018; ECDC, 2019). Rates are usually lower (≤ 3%) in the Netherlands and Scandinavia, possibly due to the aggressive infection control campaign (ECDC, 2019). In 2019, MRSA infections were associated with the death of more people than HIV/AIDS and tuberculosis combined (Murray et al., 2022).

MRSA was first reported in Australia from a Sydney hospital in 1965 (Rountree & Beard, 1968). Subsequently, MRSA isolates were sporadically detected in hospitals throughout the country for several years (Turnidge & Bell, 2000). Currently, healthcare-acquired MRSA (HA-MRSA) strains resistant to flucloxacillin and first-generation cephalosporins are commonly found in many parts of the country (AURA, 2021). High rates of resistance to erythromycin and ciprofloxacin, and moderate rates of resistance to clindamycin, gentamicin, and trimethoprim-sulfamethoxazole are generally associated with HA-MRSA (AURA, 2021). In Australia, more than 81-87% of *S. aureus* isolates were found to be resistant to benzylpenicillin in 2018-2019, and 17-19% of isolates from blood and other specimens were found to be resistant to oxacillin (methicillin) (AURA, 2021).

HA-MRSA clones commonly exhibit the MDR phenotype, and treatment depends mainly on reserve antimicrobials, such as vancomycin, rifampicin, teicoplanin, and fusidic acid (Kluytmans et al., 1997; Lina et al., 1999). However, the vancomycin-intermediate *S. aureus* (VISA) resistant to teicoplanin was isolated in Japan in 1996 and has since been reported throughout the world (Lina et al., 1999). Additionally, vancomycin-resistant *S. aureus* (VRSA), although less common than VISA, was reported in 2002 capable of resisting both vancomycin and teicoplanin (Kluytmans et al., 1997; Lina et al., 1999; Appelbaum, 2007). Although infection associated with MRSA is often manageable, this bacterium has an outstanding ability to emerge and spread in different epidemiological settings challenging the infection control system that historically targets only HAI (Fluit, 2012; Mediavilla et al., 2012).

1.4 Emergence and dissemination of antibiotic resistance in general communities

The wider healthcare settings have been the major area of concern for antibiotic resistance due to the high consumption of antibiotics, the high presence of pathogens, and poor hygiene practices that contributed to the spread of antibiotic-resistant bacteria within and between patients (Roca et al., 2015). However, the emergence and spread of antibiotic resistance have also been found in the general community, which has received less attention until recently (Levy, 1997; Levy, 2002; Ho et al., 2007; Ho et al., 2009). During the last few decades, a steady increase in antibiotic resistance has been observed in the community, particularly resistance to quinolones, vancomycin, third-generation cephalosporins, and carbapenems (van Duin & Paterson, 2016). Studies like van Duin and Paterson (2016) on antibiotic resistance in the community have evaluated a small proportion of the community who are sick and seek medical support. However, the spread of antibiotic resistance into the non-patient community and the level of resistance in common human pathogens in the community is not well understood.

Like healthcare settings, several studies have found that antibiotic use has been excessive and inappropriate in the community, which facilitates the development of resistance (Arason et al., 1996; Melander et al., 2000; Spellberg et al., 2008; Rather et al., 2017; Godman et al., 2020). According to the European Surveillance of Antimicrobial

Consumption Network (ESAC-Net), the systemic consumption of antimicrobials in the community in 2019 ranged from 34.1 (Greece) to 9.5 (the Netherlands) DDDs per 1,000 inhabitants per day (ECDC, 2020). In Australia, the rate of antimicrobial use is high compared to most European countries, and most antibiotic use occurs outside of the hospital in the community, including general practice, residential aged care facilities, and dental clinics (AURA, 2021). Compared to 30 European countries and Canada, Australia ranked seventh highest in terms of antimicrobial use in the community, with an estimated 22.9 DDDs per 1,000 inhabitants per day in 2019 (AURA, 2021). Inappropriate use of antibiotics in the community usually occurs in the prescribing for viral infections and self-medication (Rather et al., 2017; Godman et al., 2020). Several studies have identified a lack of knowledge in general communities about the inaction of antibiotics against viruses, the adverse effects of antibiotics, and poor awareness of antibiotic resistance (Eurobarometer, 2010; McNulty et al., 2007). In the United States, approximately 28% of unnecessary antibiotic courses were prescribed in the outpatient setting in 2014-2015, mostly for acute respiratory conditions caused by viruses, such as colds, bronchitis, and sore throats, and even for sinus and ear infections (Hersh et al., 2021). Although guidelines in Australia recommend that antibiotics are not indicated as routine therapy for upper respiratory tract infections, a large proportion of patients who presented to a general practitioner (GP) were reported to have received an antimicrobial for these conditions (AURA, 2021). For example, no justification was recorded for 81.5% of GP prescriptions for acute bronchitis and 80.1% for acute sinusitis in 2019 (AURA, 2021).

Several other factors facilitate the emergence and spread of antibiotic resistance within the community, such as ineffective hygiene and sanitation; lack of access to clean water, assured quality antibiotics and diagnostics; use of antibiotics in agriculture; quality of governance; migration and travel (Larson, 2007; Holmes et al., 2016; Collignon et al., 2018). In addition, discharge into the community of patients from healthcare settings with antibiotic exposure and social networks of individuals in the community (e.g. households, schools, and childcare facilities) have been reported to contribute to the development and spread of resistance beyond healthcare settings (Furuya & Lowy, 2006).

The prevalence of ESBL-E, CRE, VRE, and MRSA has been increasing in community-acquired or community-associated infection (CAI) (Kelly et al., 2017; van Duin &

Paterson, 2020). CAI is defined as an infection contracted outside of a healthcare setting or diagnosed within 48 hours of hospital admission without any previous healthcare encounter (Friedman, 2002). The following section provides an overview of the epidemiology and distribution of these critically important pathogens in community settings.

1.4.1 Epidemiology and distribution of ESBL-E associated with CAIs

The emergence of community-acquired (CA) ESBL-EC infections has been reported since the early 2000s in multiple countries, mainly in Europe and Canada (Rodríguez-Baño et al., 2004; Pitout et al., 2007; Woodford et al., 2007). In each of these cases, CTX-M-type ESBLs (i.e. CTX-M-15) are associated with CAIs other than historically common TEM- and SHV-types (Bush & Fisher, 2011). After the initial occurrence in the 2000s, CA-ESBL-producing organisms have also been observed in other regions of the world, including South America, East Asia, and Oceania (Apisarnthanarak et al., 2007; Minarini et al., 2007; Kariuki et al., 2007; Moor et al., 2008; Yumuk et al., 2008; Kang et al., 2012). CA-UTI caused by ESBL-EC was first reported in the United States in 2007 (Doi et al., 2013). Subsequently, CA-ESBL-EC infections have been reported in various parts of the United States (Freeman et al., 2009; Doi et al., 2013). For example, data obtained from community hospitals in North Carolina between 2005 and 2008 found that 27.2% of UTIs were associated with ESBL-EC (Freeman et al., 2009). In another multicenter study during 2009–2010 in the United States, 4% of CA-EC isolates were reported as ESBL producers, where the most common ESBLs identified were CTX-M-type (91%), and the remaining were SHV- (8%) or cephamycinase-2 (CMY-2)-types (1%) (Doi et al., 2013). Half of these isolates (54%) belonged to the globally disseminated ST131 MDR clone (Doi et al., 2013), characterised by CTX-M-type ESBL production, high virulence gene content, resistance to fluoroquinolones, and co-resistance to aminoglycosides and trimethoprim-sulfamethoxazole (Petty et al., 2014).

CA-ESBL-EC infections are particularly frequent in Asia, South America, the Middle East, and some parts of Europe, while a lower prevalence was reported in Australia, New Zealand, North America, and some parts of Northern Europe (van Duin & Paterson, 2020). Various studies have found several specific risk factors for CA-ESBL-EC in these low-prevalence regions. A case-control study in Chicago identified risk factors for ESBL-

EC, including prior use of ciprofloxacin, travel to India, and increasing age (Banerjee et al., 2013). Similarly, a population-based survey in London reported older age and South-Asian ethnicity as risk factors for ESBL-EC bacteriuria (Rao et al., 2015). Likewise, in Australia and New Zealand, birth on the Indian subcontinent, UTIs in the past year, and travel to Southeast Asia, India, China, Africa, or the Middle East were risk factors for CAIs with third-generation cephalosporin-resistant *E. coli* (Rogers et al., 2014). According to Australian national data, 3.2% of community isolates of *E. coli* and 3.2%–4.0% of *Klebsiella spp.* were resistant to third-generation cephalosporin (i.e. ceftriaxone) (Turnidge et al., 2013), and resistance to this antibiotic has been reported to be increasing in recent years (AURA, 2021).

Hypervirulent strains of *K. pneumoniae* (hvKP), also called 'hypermucoviscous', have become a significant health problem in Asia (Ko et al., 2002; Li et al., 2014). These strains were first described in Taiwan in 1986 from a clinical syndrome of CA *K. pneumoniae* infections (Liu et al., 1986). The hvKP strains have several unique features, including association with CA pyogenic liver abscess in healthy individuals and metastatic infections, including meningitis (Ko et al., 2002). Although most of these strains exhibit susceptibility to multiple antibiotics, their resistance and prevalence appear to be increasing globally (Li et al., 2014).

1.4.2 Epidemiology and distribution of CRE associated with CAIs

A high degree of variability in the occurrence of CRE in community settings was found between studies (Rai et al., 2014; Tang et al., 2016; Kelly et al., 2017). A systematic review of the literature found variability in reported data ranging from 5.6 to 10.8% in the USA and 7.7 to 29.5% worldwide on the prevalence of community-acquired CRE (CA-CRE) isolates (Kelly et al., 2017). The highest percentage of CA-CRE was reported from Asia, particularly in Taiwan (29.5%) and India (9.9%) (Rai et al., 2014; Tang et al., 2016). Plasmid-borne carbapenemases have been reported in food-producing animals in China, and carbapenemase-producing bacteria have been found in drinking water in India (Walsh et al., 2011; Liu et al., 2017; Pulss et al., 2017). These additional reservoirs of CRE may drive community-associated epidemics with the continued movement of resistant organisms beyond the healthcare setting. The silent spread of CRE through asymptomatic colonisation added to the global concern about the containment of these

bacteria. In 2016, active surveillance in a United States hospital using perirectal swabs identified six asymptomatically colonised patients with VIM-type CRE (Yaffee, 2016). In Australia, the percentage of CRE was low (0.04%) in outpatient UTIs (Turnidge et al., 2013). The endemicity of IMP-type CRE is low in Australia, and there is no evidence of the establishment of other types of carbapenemase (AURA, 2021). As *Enterobacteriaceae* are a common cause of CAIs and ESBL-producing Gram-negative bacteria are widespread in the community, the CDC warns that CRE could follow a similar genetic mode of transmission and spread in the community (Salles et al., 2013; CDC, 2013; Ebrahimi et al., 2014; CDC, 2015).

1.4.3 Epidemiology and distribution of VRE associated with CAIs

Studies in the 1990s did not detect VRE in subjects without healthcare exposure in the United States (Coque et al., 1996; Silverman et al., 1998). In contrast, VRE was detected in stool samples from healthy volunteers in a European study in the same time period (Bruinsma et al., 2003). The use of avoparcin (a glycopeptide antibiotic) in food animals in Europe was the underlying reason for this difference in VRE infections between Europe and the United States (Klare et al., 1999; Bruinsma et al., 2003). Avoparcin was widespread in Europe up to 1997 and was never approved for use in the United States (Klare et al., 1999). When avoparcin was banned for use in animal food production, VRE rates in human volunteers and animal samples decreased (Klare et al., 1999; Bruinsma et al., 2003). These findings demonstrate the critical link between antibiotic use in the food industry and resistance rates in humans. Community-acquired VRE (CA-VRE) infections began to appear in the United States around 2000 (Cetinkaya et al., 2000). In an ambulatory care clinic in Nashville, Tennessee, three of 100 subjects were found to be colonised with VRE, of whom one patient had no exposure to a healthcare setting or antibiotics (Raja et al., 2005). VRE was also isolated from wastewater from a semi-closed agri-food system in Texas (Poole et al., 2005). Furthermore, *Enterococcus*, such as the VRE, has been found to be persisted for several days to weeks in wastewater (Goldstein et al., 2014; Young et al., 2016; Zaheer et al., 2020).

In Australia, the majority of enterococcal bacteraemia episodes (52.9%) in 2019 were acquired from the community that were more frequently caused by *E. faecalis* (69.7%) than *E. faecium* (30.2%) (Coombs et al., 2020a). VRE has become a problem in Australia,

with only two or three reserved antimicrobials currently available to treat serious infections (AURA, 2021). In addition, the polyclonal emergence of *vanA* VRE has recently been reported in Australia, which has replaced *vanB* VRE in some Australian centres since 2012 (Coombs et al., 2014; Coombs et al., 2020a). However, factors associated with this epidemiological change remain unknown, and future studies are required to investigate potential community sources to control the increased incidence of VRE in Australia.

1.4.4 Epidemiology and distribution of MRSA associated with CAIs

MRSA infection had been primarily considered to be associated with healthcare, however, in the past two decades a transition of MRSA from healthcare settings to the community has occurred with the emergence of community-acquired MRSA (CA-MRSA) in many parts of the world, involving a small number of unique MRSA clones (Mulvey et al., 2005; Gilbert et al., 2006). CA-MRSA is associated with skin and soft tissue infections, such as furunculosis, pustulosis, and abscesses (Mulvey et al., 2005; David & Daum, 2010). Fatal necrotising pneumonia and invasive disease have also been reported (Adam et al., 2007). CA-MRSA contains Staphylococcal cassette chromosome *mec* (SCC*mec*) types IV or V, which usually produces Panton-Valentine leukocidin toxin (PVL) (Vandenesch et al., 2003). PVL is associated with fatal necrotising pneumonia and severe skin sepsis and is typically susceptible to most non-β-lactam antibiotics (Vandenesch et al., 2003). Compared to HA-MRSA infections, CA-MRSA infections are of particular concern due to their rapid emergence (Leclercq, 2009), increased prevalence (Larsen et al., 2009), enhanced virulence (Herold et al., 1998), and the ability to cause serious infections in otherwise healthy individuals (Hidron et al., 2009). With their introduction into healthcare settings, CA-MRSA strains can cause surgical site infections, hospital-acquired bacteraemia, and outbreaks in hospital nurseries and maternity units (Otter & French, 2006).

CA-MRSA infections were first reported in the remote population of Western Australia in the early 1990s (Udo et al., 1993), and in the United States associated with cases of fatal infections in children in North Dakota and Minnesota at the end of the 1990s (Herold et al., 1998; Gorak et al., 1999; Naimi et al., 2001). Subsequently, CA-MRSA infections have also been reported in many parts of the world, including the Middle East, Europe,

Asia, and Oceania (Okuma et al., 2002; Vandenesch et al., 2003; Wannet et al., 2004; Denis et al., 2005; Harbarth et al., 2005; Witte et al., 2005). Since their first emergence in Australia, CA-MRSA clones have diversified and increased in prevalence. The Queensland clone (ST93-IV [2B]) of CA-MRSA has been the dominant clone in Australia since its first detection in southern Queensland in 2000 (Nimmo & Coombs, 2008). Subsequently, it has spread to become dominant in the Northern Territory, South Australia, and at the same rates as the WA-1 clone in Victoria and New South Wales (Coombs et al., 2016). This CA-MRSA clone is usually susceptible to non-β-lactam antibiotics and can produce PVL, but the reason for its rapid emergence and spread remains unclear. In 2019, the Queensland CA-MRSA clone represented 24.4% of all CA-MRSA in Australia (Coombs et al., 2020b). In Australia, β-lactams are usually the treatment of choice for staphylococcal infections (AURA, 2021). Therefore, CA-MRSA strains are likely to cause failure in the treatment with β-lactams (i.e. flucloxacillin and cefalexin) commonly prescribed by community practitioners, resulting in hospitalisation for treatment with parenteral antibiotics.

1.5 The potential of wastewater-based epidemiology to monitor antibiotic resistance in the community

The emergence and spread of MDR bacteria require a global and local effort to address this problem (WHO, 2014). To control the ever-increasing rate of AMR, the WHO launched the Global Antimicrobial Resistance Surveillance System in 2015 to promote worldwide surveillance of AMR trends in priority pathogens and detection of emerging resistance, mainly in healthcare settings (WHO, 2015). The Australian Government responded to the WHO call for action with the National Antimicrobial Resistance Strategy 2015-2019 (Australian Department of Health, 2015), and the Australian Commission for Safety and Quality in Health Care began a nationwide coordinated and comprehensive surveillance system for Antimicrobial Use and Resistance in Australia (AURA, 2016). AURA reports data on AMR in priority pathogens and antimicrobial use (AMU) annually at the national level, collected almost exclusively from healthcare settings (AURA, 2016).

A major limitation of current AMR surveillance in Australia and other parts of the world is the almost exclusive focus on healthcare settings that only surveys a small healthcare-

engaged part of the population but not the entire community (WHO, 2015; AURA, 2016). Community factors, such as overuse and misuse of antibiotics in the community (see Section 1.4 for details), and the use of antibiotics in agriculture and animal husbandry, however, contribute significantly to the emergence of resistance in non-healthcare settings (Ball et al., 2004; Levy & Marshall, 2004; Klugman & Lonks, 2005). In addition to antimicrobial use, several economic, cultural, and social determinants have also been reported to facilitate the emergence and worldwide dissemination of antibiotic-resistant bacteria and resistance genes (Holmes et al., 2016; Collignon et al., 2018). Therefore, without data on community-based AMR and its contributing factors, the control of AMR will not be evidence-based. Surveillance of AMR in the general community by obtaining samples from a large representative group of predominantly healthy people is impractical due to associated costs and ethical constraints (Bauer, 2008). Wastewater-based surveillance or wastewater-based epidemiology (WBE) is a potential alternative to simply, reliably, and continuously monitor AMR trends in the community.

WBE was proposed in 2001 as a diagnostic tool to track community traits such as illicit drug use (Daughton, 2001). The approach is based primarily on the assumption that any chemical or biological materials that are excreted by humans and remain stable in wastewater can be used to back-calculate the initial concentrations excreted by the serviced population located within the wastewater treatment plant (WWTP) catchment areas (Figure 1.1) (Choi et al., 2018; Sims & Kasprzyk-Hordern, 2020). Currently, WBE has been successfully used to monitor the use of illicit drugs and pharmaceuticals within a population (van Nuijis et al., 2011; Ort et al., 2014; Lai et al., 2016; Causanilles et al., 2017; Choi et al., 2018). The potential of wastewater testing for viral surveillance has also been discussed in the literature (Barras, 2018; O'Brien & Xagoraraki, 2019; Wigginton et al., 2015). For example, wastewater analysis was used to monitor poliovirus circulation within populations and the efficacy of immunisation against it during the global polio eradication program (Hovi et al., 2012; Roberts, 2013; Ndiaye et al., 2014). In addition, wastewater-based surveillance has been used for retrospective prediction of hepatitis A and norovirus outbreaks (Hellmér et al., 2014). More recently, WBE has also been used successfully and extensively as an early warning system for the prevalence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in the community located within a

WWTP catchment (Ahmed et al., 2020; Gonzalez et al., 2020; La Rosa et al., 2020; Wu et al., 2020).

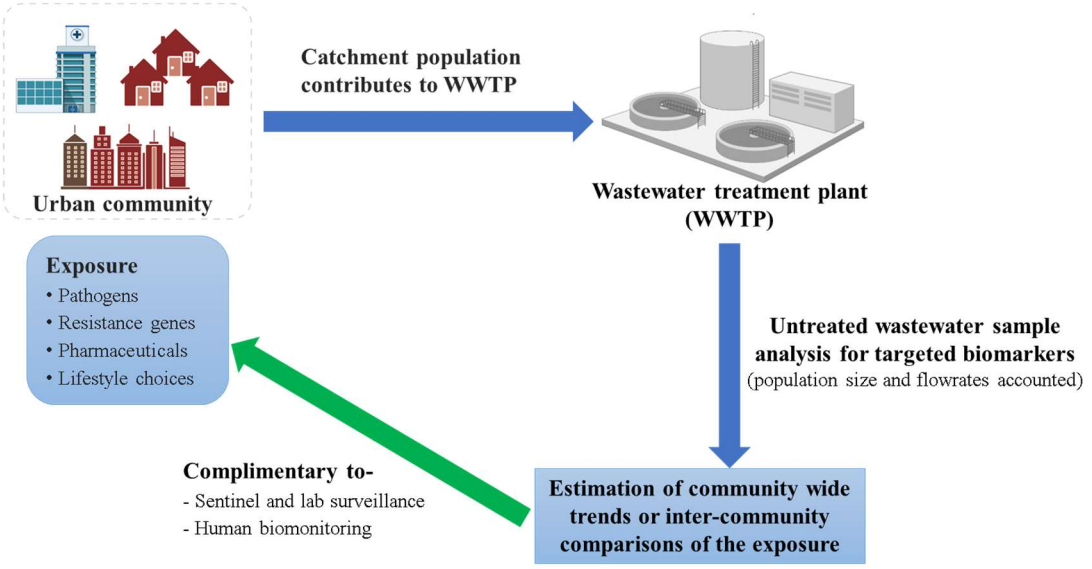


Figure 1.1 Illustration of the wastewater-based epidemiology (WBE) concept.

Previous studies also substantiate the idea that bacteria in wastewater are good representatives of the individual's indigenous microbiota (Kühn et al., 2003; Reinthaler et al., 2013). For example, a multi-centre European study on enterococci isolated from humans, animals, the environment, wastewater, and manure found that the enterococcal population structure in wastewater resembled that of a large population of individual faecal samples from humans and animals with respect to species distribution, clonal diversity, and population similarities (Kühn et al., 2003). In addition, Reinthaler et al. (2013) found that resistance rates among *E. coli* in urban wastewater correspond well to the increasing resistance rates of human clinical isolates. Hence, wastewater testing has emerged as an attractive means for different population-based surveillance of the prevalence and trends of antibiotic resistance in human pathogens.

WBE has been used successfully to monitor the prevalence of antibiotic resistance in a selection of specific human pathogens in Israel (Meir-Gruber et al., 2016). Using a modified plate count technique, Meir-Gruber et al. (2016) identified several pan-resistant bacteria, including CRE, VRE and MRSA, in hospitals and community wastewater collected throughout Israel. In the Netherlands, municipal wastewater surveillance has been used to reveal the distribution of carbapenemase-producing *Enterobacteriaceae*

(CPE) in the population at a national level (Blaak et al., 2021). However, wastewater samples were analysed only at one time-point in this study and, therefore, only represented a 'snap-shot' of CPE prevalence in the Dutch population during the study period (Blaak et al., 2021). In Sweden, Kwak et al. (2015) examined resistance rates among *E. coli* isolates from untreated urban wastewater and found that increasing frequencies of antibiotic resistance in *E. coli* reflected a rising trend of resistance in the community. However, this study focused on the level of resistance in *E. coli* in a single WWTP, which may not reflect overall resistance trends in the community or levels of resistance in other important pathogens. In Australia, Thompson et al. (2013) tracked the movement and survival of MRSA from hospital wastewater in the receiving WWTP and their presence in discharged effluent. However, this study did not assess the prevalence of MRSA or other priority pathogens from WWTP of general communities. Recently, metagenomic sequencing of untreated urban wastewater from 60 countries found significant and systematic variations in the abundance and diversity of antibiotic resistance genes around the world, driven mainly by socioeconomic and environmental factors (Hendriksen et al., 2019). However, the study only analysed a single WWTP per country at a single time point and, therefore, could not discover spatial or temporal trends. Therefore, a comprehensive understanding of the spatio-temporal development of antibiotic resistance in priority pathogens and factors driving their dissemination in the wider community is pivotal to control and to develop effective interventions.

1.6 Thesis Aims

Current healthcare infection surveillance has provided significant information on the emergence and spread of resistance in priority pathogens in clinical settings. However, the spatio-temporal development of resistance in important pathogens and potential contributing factors that facilitate their emergence and spread beyond clinical settings remained largely unexplored. This results in significant and persistent gaps in our ability to fully understand and manage the spread of antibiotic resistance. Wastewater-based surveillance is a reliable and affordable alternative to monitor the community for antibiotic resistance trends in priority pathogens. This thesis aimed to establish routine wastewater-based surveillance as a mass-population monitoring approach for geospatial-temporal trends of antibiotic resistance in various priority pathogens and to evaluate its value to understand their emergence and distribution in the general community. Another

objective of the thesis was to understand the nature and origin of wastewater isolates and the mechanisms underlying their resistance. The specific aims for each chapter are as follows.

Chapter 2 investigates whether regular wastewater-based monitoring could establish a baseline for a selection of clinically important AMR pathogens in the general community of Sydney and whether the load of AMR pathogens in wastewater could be explained by demographic, socioeconomic, and healthcare infection-related parameters. I focused on ESBL-E*,* CRE, VRE, and MRSA due to their clinical importance. These priority pathogens load was mapped onto the Sydney urbanised area to monitor their geospatial-temporal trends and correlated with selected parameters to identify factors that could explain their observed distribution and variation. The data generated in this chapter lead to a broader project in which another researcher will analyse the global patterns of antibiotic resistance. In the following two chapters, I looked at very specific resistance patterns.

Chapter 3 used whole-genome sequencing to define the characteristics of ESBL-producing and carbapenem-resistant *E. coli* isolated from wastewater. These isolates were selected based on their resistance to a range of antibiotics that were critically important to human health. Genomic and phylogenetic analysis was used to identify their relationship with known *E. coli* lineages, the genetic bases of their resistance, as well as virulence, and their potential for the acquisition and spread of resistance and virulence-encoding genes.

Chapter 4 covers the genomic characterisation of ESBL-producing, and carbapenem-resistant *K. pneumoniae* and *K. variicola* strains isolated from wastewater. The isolates were chosen because of their resistance to various critically important antibiotics for human health. The aim was to identify their phylogenetic relationship with known *Klebsiella* lineages, the molecular mechanisms underpinning resistance, the potential for virulence, and their potential to acquire and disseminate resistance genes.

Chapter 5 includes a summary and a general discussion of the work presented in this thesis.

Chapter 2 Wastewater-based monitoring reveals geospatial-temporal trends for antibiotic-resistant bacteria in a large urban community

2.1 Introduction

Antimicrobial resistance (AMR) is recognised as a significant global public health threat, with the potential of once-treatable infections becoming untreatable (WHO, 2014). It has been estimated that AMR was directly related to approximately 1.27 million deaths worldwide in 2019 (Murray et al., 2022). Therefore, both global and regional efforts are focused on controlling the ever-increasing rate of AMR to preserve the efficacy of available antibiotics for future generations (WHO, 2015). Increasing concerns about AMR led the World Health Organization (WHO) to launch the Global Antimicrobial Resistance Surveillance System (GLASS) in 2015 (WHO, 2015) to support the implementation of the Global Action Plan on Antimicrobial Resistance (GAP-AMR) (WHO, 2015). GLASS aims to foster worldwide surveillance of AMR trends in priority pathogens and the detection of emerging resistances (WHO, 2015). However, the focus of GLASS is mostly on AMR in healthcare settings (WHO, 2015). Aligned with GAP-AMR, the Australian Government adopted the National Antimicrobial Resistance Strategy 2015-2019 (Australian Department of Health, 2015) and the Australian Commission for Safety and Quality in Health Care established a nationwide coordinated and comprehensive surveillance system for Antimicrobial Use and Resistance in Australia (AURA, 2016). AURA collects and reports data annually at national levels for AMR in priority pathogens and antimicrobial use (AMU), but almost exclusively from healthcare settings (AURA, 2021). This is a major limitation of current AMR surveillance in Australia and many other parts of the world, which only surveys a small, healthcare-engaged part of the population and not the entire community.

The exclusive focus on healthcare settings has its genesis in the perception that the unrestrained global use of antimicrobials in healthcare facilities accelerates the emergence and spread of AMR and that ameliorating inappropriate prescribing in healthcare settings will reduce AMR (Davies & Davies, 2010; Bell et al., 2014; WHO, 2015; Holmes et al., 2016; Aidara-Kane et al., 2018). However, there is no clear

correlation between reduced AMU and decreased AMR in healthcare settings (Enne et al., 2001; Sundqvist et al., 2010). Importantly, healthcare-based AMU alone only explains a minor proportion of the AMR occurrence worldwide, and several cultural, social and economic determinants that facilitate the emergence and spread of AMR and resistance genes may contribute to the increased prevalence and variations of AMR across countries (Collignon et al., 2018; Hendriksen et al., 2019). Therefore, a better capture of AMR and its potential contributing factors in the wider community is pivotal to control and to develop effective interventions.

Monitoring the general community for AMR by collecting individual faecal samples from a large representative group of the non-hospitalised human population is impractical due to costs, high refusal rates, and ethical constraints (Bauer, 2008). An alternative is testing untreated wastewater since microorganisms in wastewater reflect the microbiota of the wider community who contribute to wastewater (Kühn et al., 2003; Reinthaler et al., 2013). Wastewater-based epidemiology (WBE) is now an internationally successful discipline (Thomas & Reid, 2011; Novo et al., 2013; Chen et al., 2014; Fahrenfeld & Bisceglia, 2016; Lai et al., 2016; Karkman et al., 2018; Ahmed et al., 2020; Gonzalez et al., 2020; Sims & Kasprzyk-Hordern, 2020) and a rapidly growing approach for mass-population surveillance of endogenous and exogenous biomarkers of the community located within catchment areas of wastewater treatment plants (WWTPs).

This study reports on wastewater testing as a simple, reliable and affordable mass-population monitoring method for repeat testing of AMR to provide a temporally resolved analysis of the general community. I used Sydney, New South Wales (NSW), Australia, as a model urbanised area to establish a wastewater-based surveillance of the general community. I focused on extended-spectrum β-lactamases-producing *Enterobacteriaceae* (ESBL-E), carbapenem-resistant *Enterobacteriaceae* (CRE), vancomycin-resistant enterococci (VRE), and methicillin-resistant *Staphylococcus aureus* (MRSA) because of their clinical importance. These priority pathogens load was geospatially mapped onto the Sydney urbanised area and correlated with selected demographic, socioeconomic and healthcare infection-related parameters to identify factors that could explain their observed distribution and variation.

2.2 Materials and Methods

2.2.1 Sample collection

Untreated, influent wastewater samples were collected from 25 WWTPs around Sydney (Figure 2.1). Grab samples were collected in 500 ml sterile bottles by the utility Sydney Water, a major supplier of water, wastewater, recycled water and some stormwater services to the Greater Sydney region. Field samplers from Sydney Water collected samples from each site in July 2017, February 2018, May 2018, September 2018, February 2019, and June 2019. Most of the sampling occurred before 12 pm, with the occasional from 1 pm to 2 pm, and all occurred during dry days. The samples were then transported below 7˚C to the UNSW laboratory within 24 hours of collection for preparation and analysis.

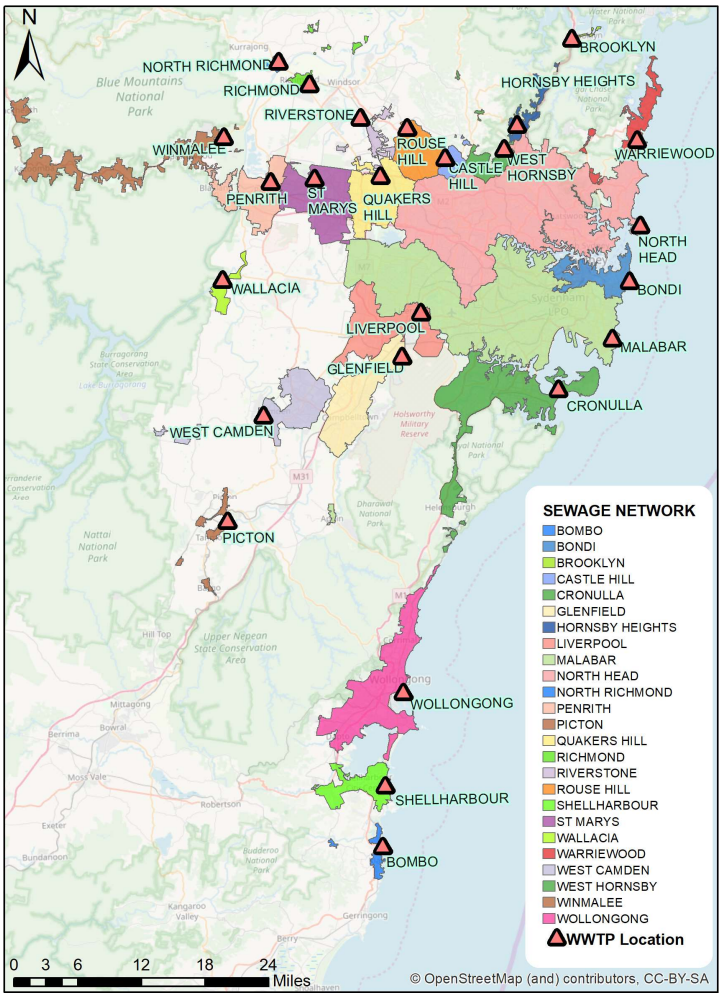


Figure 2.1 Location of the 25 wastewater treatment plants (WWTPs) and the respective catchment areas in Sydney, Australia.

2.2.2 Isolation and enumeration of total cultivable and AMR bacteria

To determine the colony-forming units per millilitre (CFUs/ml), the wastewater was thoroughly homogenised and then serially diluted (1:10 to 1:100000) with 0.9% sterile saline solution. Aliquots of 100 µl from each dilution were spread evenly onto sterile nutrient agar plates (Oxoid, Hampshire, England) and incubated under aerobic conditions at 37˚C for 24 hours to estimate total bacterial counts. To determine the CFUs/ml of resistant bacteria, serial dilutions of 1:10 and 1:100 were spread (100 µl) onto different types of ChromID agars specific for ESBL-E, CRE, VRE and MRSA (bioMérieux, Marcy I'Etoile, France). These selective and indicator ChromID plates were incubated under aerobic conditions between 24 and 48 hours at 37˚C. Colonies were qualitatively assessed based on their colour, following the manufacturer's instructions (bioMérieux). The **s**uspected ESBL-producing *E. coli* colonies on ChromID ESBL agar appeared as pink to burgundy; KESC (*Klebsiella spp., Enterobacter spp., Serratia spp., Citrobacter spp.*) appeared as green-blue to brown-green; and Proteeae (*Proteus spp., Providencia spp., Morganella spp.*) appeared as dark to light brown (see Text S2.1 in Supplementary Information for more details). The suspected carbapenem-resistant *E. coli* appeared as pink to burgundy, whereas KESC (*Klebsiella spp., Enterobacter spp., Serratia spp., Citrobacter spp.*) green-blue to bluish-grey or purple on ChromID CARBA SMART agar (Text S2.1). The suspected vancomycin-resistant *E. faecium* and *E. faecalis* species appeared violet and green-blue, respectively, on ChromID VRE agar (Text S2.1). The suspected MRSA colonies appeared as green on ChromID MRSA agar (Text S2.1). Each distinct-coloured colony on the ChromID agar plates with between 10 and 300 colonies (either the 1:10 or 1:100 dilution) was counted for each sample. Then, a single representative colony from each characteristic colour was selected for confirmation of bacterial identification and resistance, as described in the following sections. Finally, an estimate of CFUs/ml of wastewater was obtained for each antibiotic-resistant bacterium from the number of positive-coloured colonies on ChromID agar, the dilution factor, and the volume plated (see Text S2.1 for details). The limit of detection (LOD) of each antibiotic-resistant bacterium was calculated from the lowest number viable colonies recovered by plating 100µl of one-fold diluted wastewater sample on the respective ChromID plates. The LOD for each of the four pathogens was 10^2 CFUs/ml across all sampling sites and periods. The CFUs/ml of resistant bacteria were divided by the

CFUs/ml of the total bacteria to provide a relative proportion and then normalised by the daily flow rates of the respective WWTP to adjust for different flow rates so that the result from each of the 25 wastewater sites could be compared. The resulting parameter was referred to as the flow normalised relative (FNR) antibiotic-resistant bacterial load.

2.2.3 Confirmation of bacterial identification

Single colonies with distinct features from ChromID plates were sub-cultured onto horse blood agar (HBA) plates (bioMérieux). Pure colonies from the HBA were identified by matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS) either at the pathology laboratory of the NSW Health Pathology Liverpool Hospital or at the Bioanalytical Mass Spectrometry Facility (BMSF) at UNSW Sydney. MALDI-TOF MS testing was performed following the direct colony extraction technique described elsewhere (Mellmann et al., 2008). Briefly, an individual bacterial colony was directly spotted in duplicate onto a steel Bruker MALDI-TOF target plate (96 spots), overlaid with 1µl of matrix solution (α -cyano-4-hydroxycinnamic acid dissolved in 50 % acetonitrile and 2.5 % trifluoroacetic acid; HPLC grade; Bruker Daltonics). The mass spectra were generated using a Bruker MicroFlex LT mass spectrometer (Bruker Daltonics, Billerica, MA, USA) and analysed using the MALDI Biotyper automation control and the Bruker Biotyper 3.1 software. This Bruker software matched the target spectra with a reference spectral database of more than 3,000 organisms for identification. Following MALDI-TOF testing, all identified colonies were grown in Luria-Bertani (LB) broth (Oxoid Ltd., Basingstoke, Hampshire, UK) and stored with 30% glycerol at -80˚C for further testing.

2.2.4 Confirmation of resistant phenotypes

The antibiotic susceptibility testing (AST) of the identified bacterial isolates was performed by the VITEK 2 system (bioMérieux, Vitek-Australia Pty Ltd) in accordance with the manufacturer's instruction either at the pathology laboratory of the NSW Health Pathology Liverpool Hospital or the UNSW laboratory facility. Frozen strains were grown on HBA plates at 37˚C for 18 to 24 hours, and the pure cultures were resuspended in 3.0 mL of 0.45% sterile saline solution. The turbidity was adjusted to the equivalent of a 0.5 to 0.63 McFarland standard using the VITEK 2 Densichek instrument (bioMérieux). The test tube was then placed into a cassette, and the bacterial suspension was transferred

into the AST card with various antimicrobials dilutions using the VITEK 2 system. The susceptibility testing of Gram-negative and Gram-positive bacteria was performed using VITEK 2 AST-N246 and AST-P612 cards (bioMérieux), respectively. The AST cards were automatically processed in the VITEK 2 system until the minimum inhibitory concentrations (MICs) were obtained. Then, the VITEK 2 advanced expert system™ (AES) software (version 8.01) recognised and matched the obtained MIC patterns with an extensive and regularly updated knowledge base of 4,000 phenotypes comprising 55,000 MIC distributions. Based on matched susceptibility patterns (Barry et al., 2003), the VITEK 2 AES algorithm determines specific resistance phenotypes in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute, 2018). Please, see Text S2.1 in Supplementary Information for more details on the selection, confirmation, and enumeration of antibiotic-resistant bacterial colonies.

2.2.5 Geographical distribution of AMR isolates

The geographical distribution of each AMR pathogen was mapped using the Environmental Systems Research Institute's geographic information systems platform ArcMap™ (ArcGIS® version 10.7.1). The wastewater network map (shapefile) with the locations and catchment areas of the 25 WWTPs was obtained from Sydney Water and imported into ArcMap. A catchment area is defined as an area that drains wastewater to a WWTP before treatment. The FNR antibiotic-resistant bacterial load of each WWTP was log-transformed to normalise data distribution and then imported as a separate layer in ArcMap. Using the Feature Project tool in ArcMap, all data layers were standardised into a single projected coordinate system (Map Grid of Australia 1994, Zone 56, Transverse Mercator). Each data layer was overlaid on the wastewater network layer using the Spatial Join tool in ArcMap. The distribution of each AMR pathogen detected during all sampling periods was then visualised on separate choropleth maps in ArcMap.

2.2.6 Data source and study variables

The FNR loads of ESBL-E, CRE, and VRE isolates at six-time points within each of the 25 WWTPs were used as dependent variables. Due to the infrequent detection of MRSA at only one or two sampling sites, it was excluded from further statistical analysis. The lack of detection of CRE and VRE isolates from several locations was not considered true

zeros due to the limit of detection (10^2 CFUs/ml) imposed by sampling and were therefore excluded from the respective data set before any statistical analysis (Blasco-Moreno et al., 2019) (Supplementary Table S2.1).

Several demographic, socioeconomic, and healthcare infection-related features of the WWTP catchments were included as independent variables (Table 2.1-2.3, Table S2.2-S2.4). The population density of each of the catchment areas was estimated in accordance with the Tscharke et al. (2019) method. Briefly, the catchment maps of the WWTPs were intersected with georeferenced population counts of the most recent Australian census (ABS, 2016) at the mesh block (MB) level in ArcMap. All MBs were completely and partially intersected with the WWTP catchment boundaries. The MBs were summed in ArcMap to calculate the total population of each of the catchments. The total population of each catchment was divided by the respective catchment area to obtain the population density per square kilometre (see Text S2.2 in Supplementary Information for more details on the estimation of wastewater catchments population density). Following the same method, the proportion of the adult population 19 to 50 years of age and over 50 years of age within each of the catchment areas were also calculated approximately at the MB level using the age (AGEP) dataset of the 2016 census.

Other demographic and socioeconomic variables of each WWTP catchment were calculated in ArcMap using the statistical area 1 (SA1) level data extracted from the Australian Bureau of Statistics (ABS, 2016). For instance, the educational institution attendee status (TYSTAP) dataset was used to estimate the proportion of the population who had completed vocational education (certificate level III, IV, and diploma) and the proportion who had completed at least high school. Median weekly personal, family and household incomes were calculated in ArcMap using the total personal income (INCP), total family income (FINF), and total household income (HIND) datasets, respectively.

The total number of schools within each catchment area was calculated in ArcMap. The school dataset was obtained from the NSW Education Data Hub (https://data.cese.nsw.gov.au/) and geocoded in ArcMap based on the X and Y coordinates, then aggregated based on the wastewater catchments using the Clip and Spatial Join geoprocessing tools,

respectively. The total number of schools was divided by the total population of the respective catchment to standardise the unit of measure per 10,000 population.

Healthcare infection-related variables encompassed a combination of public and private hospitals, residential aged care facilities (RACFs) and general practitioners (GPs) (Table 2.1-2.3, Table S2.2-S2.4). Details on the data sources, categorisation, and geoprocessing of all the healthcare infection-related variables based on wastewater catchments in ArcMap are given in Text S2.3 in Supplementary Information. Briefly, the public hospital peer groups and the private hospital-related datasets were obtained from the Australian Institute of Health and Welfare, geocoded and aggregated in ArcMap based on addresses and wastewater catchments, respectively. Hospital-related variables included: the number of all (i.e. public and private), public, and private hospitals. The categories were collapsed based on the bed numbers of hospitals that were small and closely located. Public and private hospital numbers were also combined into new categories of large, co-located large, small public and small private hospitals (see Text S2.3 for details). The data for the number of hospital admissions, acute bed days, elective surgeries, and the average length of hospital stay in the last three months were collected from the Bureau of Health Information quarterly datasets and aggregated based on WWTP catchments. The number and location of all RACFs and GPs were obtained from the Australian Institute of Health and the National Health Service Directory, respectively. The locations of the facilities were geocoded based on the X and Y coordinates and aggregated based on the wastewater catchment areas in ArcMap. The RACF dataset was categorised into large, medium and small, based on the bed numbers (see Text S2.3 for details). All the healthcare infection-related variables were modified to standardise the unit of measure per 10,000 population (Table 2.1-2.3, Table S2.2-S2.4).

2.2.7 Statistical analysis

All dependent and independent variables were assessed for normality using a histogram, Shapiro-Wilk normality test, and normal Q-Q plots in the R statistical language version 4.0.2 (R Core Team, 2020) using R-Studio version 1.3.1073 (RStudio Team, 2020). Dependent variables such as FNR ESBL-E, CRE, and VRE loads were base-10 log-transformed to obtain normal distributions (Ives, 2015). Independent variables that were base-10 log-transformed included population density per square kilometre, the number of

GPs per 10,000 population, and the number of elective surgeries per 10,000 population. The number of small public hospitals per 10,000 population was square root transformed to overcome right skewness and achieve improvement in distribution. Other independent variables followed non-normal distribution and remained skewed even after different transformation methods.

The non-parametric Spearman's rank correlation coefficient (r_s) was used to investigate the bivariate correlation between the dependent and independent variables. A correlation r_s = -1 indicates a perfect inverse relationship, while r_s = 1 indicates a perfect direct relationship, and r_s = 0 indicates no relationship between variables (Hinkle et al., 2003). Several scatterplots were generated to check whether a linear trend exists between variables. Those independent variables with a statistically significant ($p < 0.05$) association with the dependent variables were selected for regression analysis.

Several simple linear regression models were fitted to the data (Mangiafico, 2015) using the linear model function (lm) in R-Studio to identify independent variables that influenced the dependent variables. All independent variables that exhibit statistically significant association with dependent variables in simple linear regression were checked for multicollinearity before inclusion in the final multiple regression analysis (Farrar & Glauber, 1967; Schroeder et al., 1990; Tu et al., 2005). The variance inflation factor (VIF) (Belsley, 1991; Hair et al., 1998) and the collinearity diagnostic test for tolerance (Belsley et al., 1980) were used to assess collinearity between independent variables. A stepwise approach was employed to remove variables with > 10 VIF and < 0.1 tolerance values, indicating multicollinearity, using the VIF function in companion to applied regression (car) package in R-Studio. For the FNR ESBL-E load, the variables excluded from the multiple regression model included population density and the proportion of the population who had completed at least high school because of the VIF-value > 10, the number of large-sized RACFs per 10,000 population due to the correlation (r = 0.87) between the number of all RACFs per 10,000 population, and the number of small public hospitals per 10,000 population due to a large number of missing values (Table 2.1). Variables that did not reach significance in the univariate regression analysis were excluded from the model for the FNR CRE load, including the number of small public hospitals per 10,000 population due to a high VIF value (> 10), the number of co-located

large hospitals per 10,000 population because of a large number of missing values, and the proportion of the population between 19 to 50 years of age (Table 2.2). For the FNR VRE load, the number of co-located large hospitals per 10,000 population was excluded from the model due to a large number of missing values (Table 2.3).

Multiple linear regression models were built separately for ESBL-E, CRE, and VRE data with those independent variables without multicollinearity using the lm function in the R-studio software. The best regression models were selected using the forward and backward stepwise model selection method using the step function in the R-studio software. All subset regression analyses were performed to choose the best model from every possible model using the regsubsets function from the leaps package in R.

2.3 Results

2.3.1 Distribution of total bacterial count and AMR isolates

The total bacterial counts across the sampling periods and WWTPs were relatively stable, with a mean $\log_{10}$ CFU/day of 13.87 and a standard deviation of 0.84 (Figure S2.1 in Supplementary Information). ESBL-E isolates were consistently detected in 100% of the WWTP s and 100% of the wastewater samples, but at varying amounts over time (Figure 2.2). The median $\log_{10}$ FNR ESBL-E load was 5.44 (range 3.19 to 8.57).

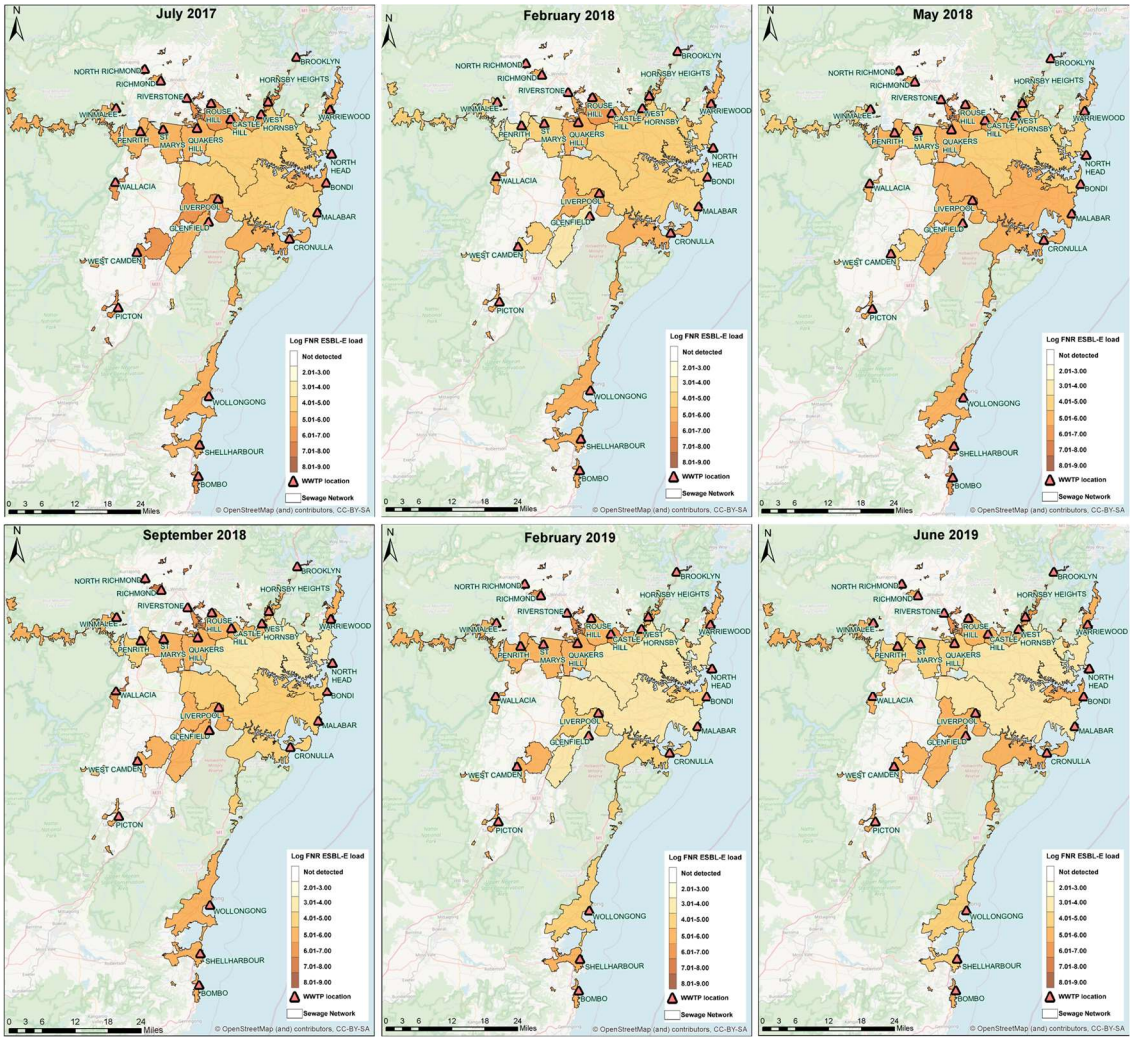


Figure 2.2 Choropleth maps showing the spatial and temporal distribution of the flow normalised relative (FNR) load of extended-spectrum β-lactamases-producing *Enterobacteriaceae* (ESBL-E) across 25 WWTP catchments in Sydney.

CRE isolates were found in 52% of the WWTPs and 18% of the samples, VRE isolates were found in 76% of the WWTPs and 26% of the samples, and MRSA isolates were found in 24% of the WWTPs and 5.33% of the samples during all sampling periods. For instance, CRE isolates were detected in seven of the 25 WWTP catchments in July 2017, one site in February 2018, two locations in May 2018, eight sites in September 2018, four locations in February 2019, and five sites in June 2019 (Figure 2.3). The $\log_{10}$ FNR CRE load ranged from 2.53 to 5.5, with a median of 4.25 for all sampling periods.

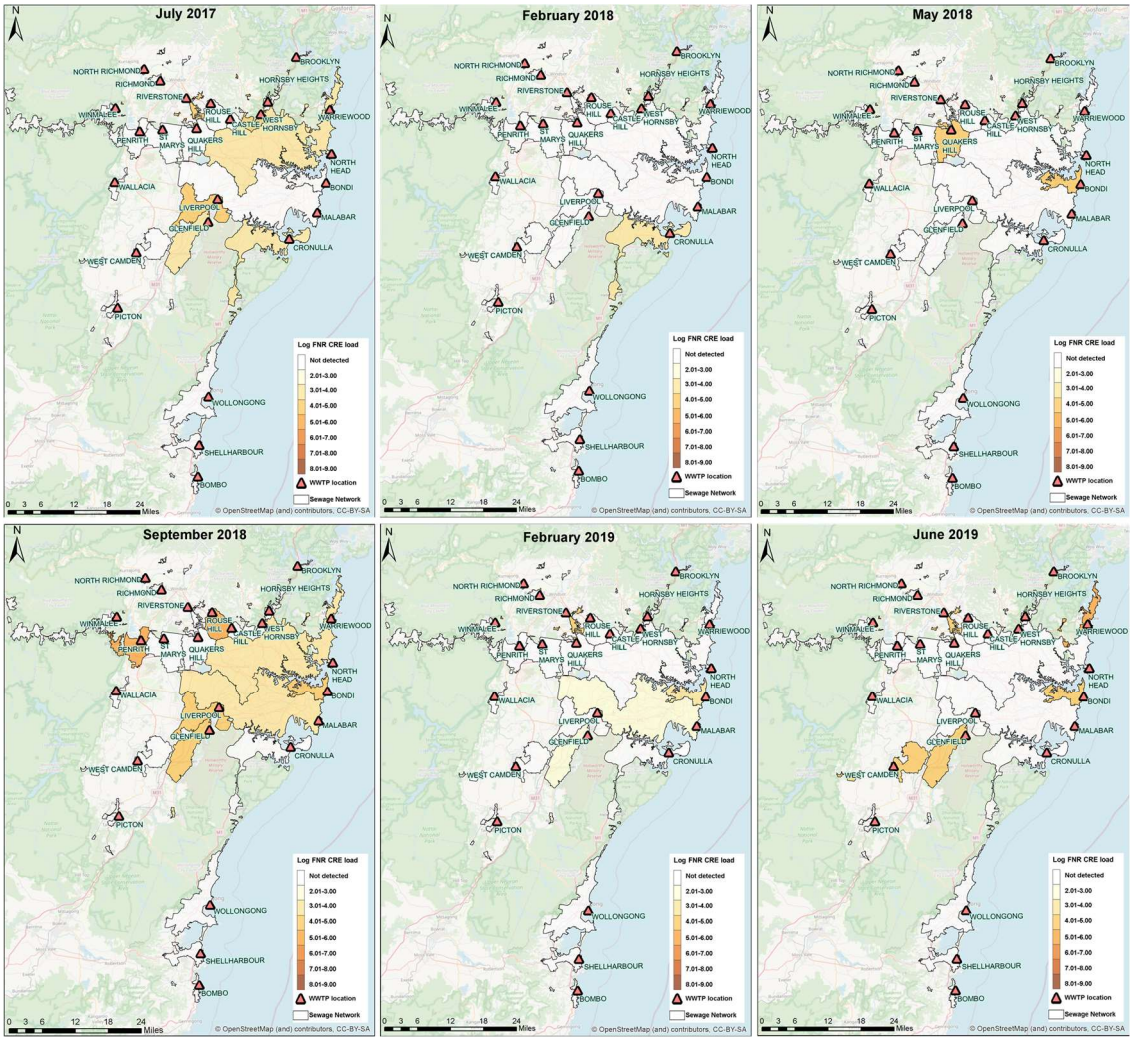


Figure 2.3 Choropleth maps showing the spatial and temporal distribution of the flow normalised relative (FNR) load of carbapenem-resistant *Enterobacteriaceae* (CRE) across 25 WWTP catchments in Sydney.

VRE isolates were found in two of the 25 WWTPs in July 2017, five locations in February 2018, nine sites in May 2018, 12 locations in September 2018, six sites in February 2019, and five locations in June 2019 (Figure 2.4). During all sampling periods, the median $\log_{10}$ FNR VRE load was 4.69 (range 2.10 to 7.25).

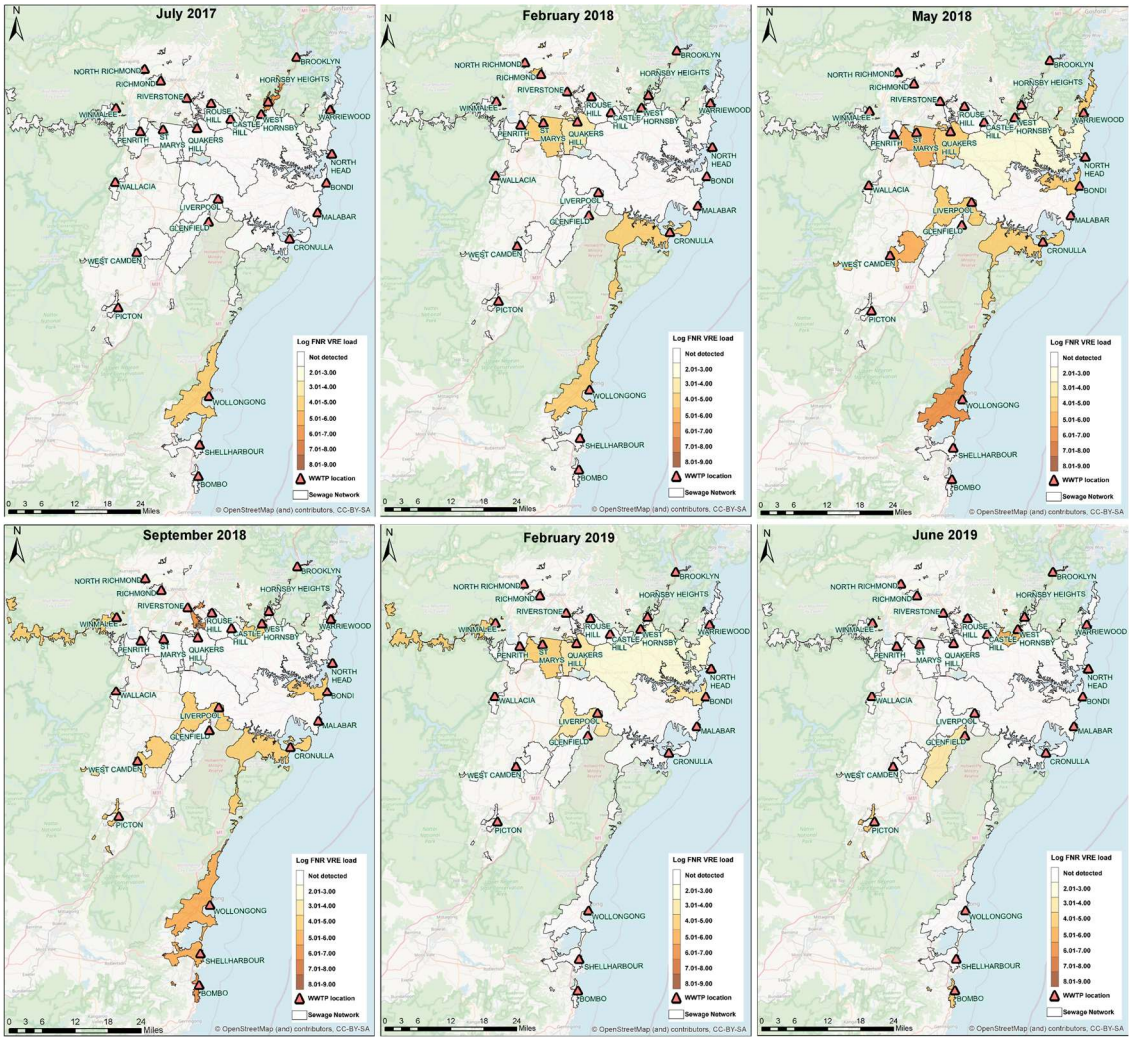


Figure 2.4 Choropleth maps showing the spatial and temporal distribution of the flow normalised relative (FNR) load of vancomycin-resistant enterococci (VRE) across 25 WWTP catchments in Sydney.

MRSA isolates were occasionally detected in the WWTP catchments during all sampling periods (Figure 2.5). MRSA was detected in the Riverstone and Rouse Hill catchments in July 2017, Richmond in February 2018, Rouse Hill in May 2018, Rouse Hill and Castle Hill in September 2018, and Warriewood and Bondi in February 2019. These locations had a median $\log_{10}$ FNR MRSA load of 4.92 (range 3.67 to 6.59) for all sampling periods.

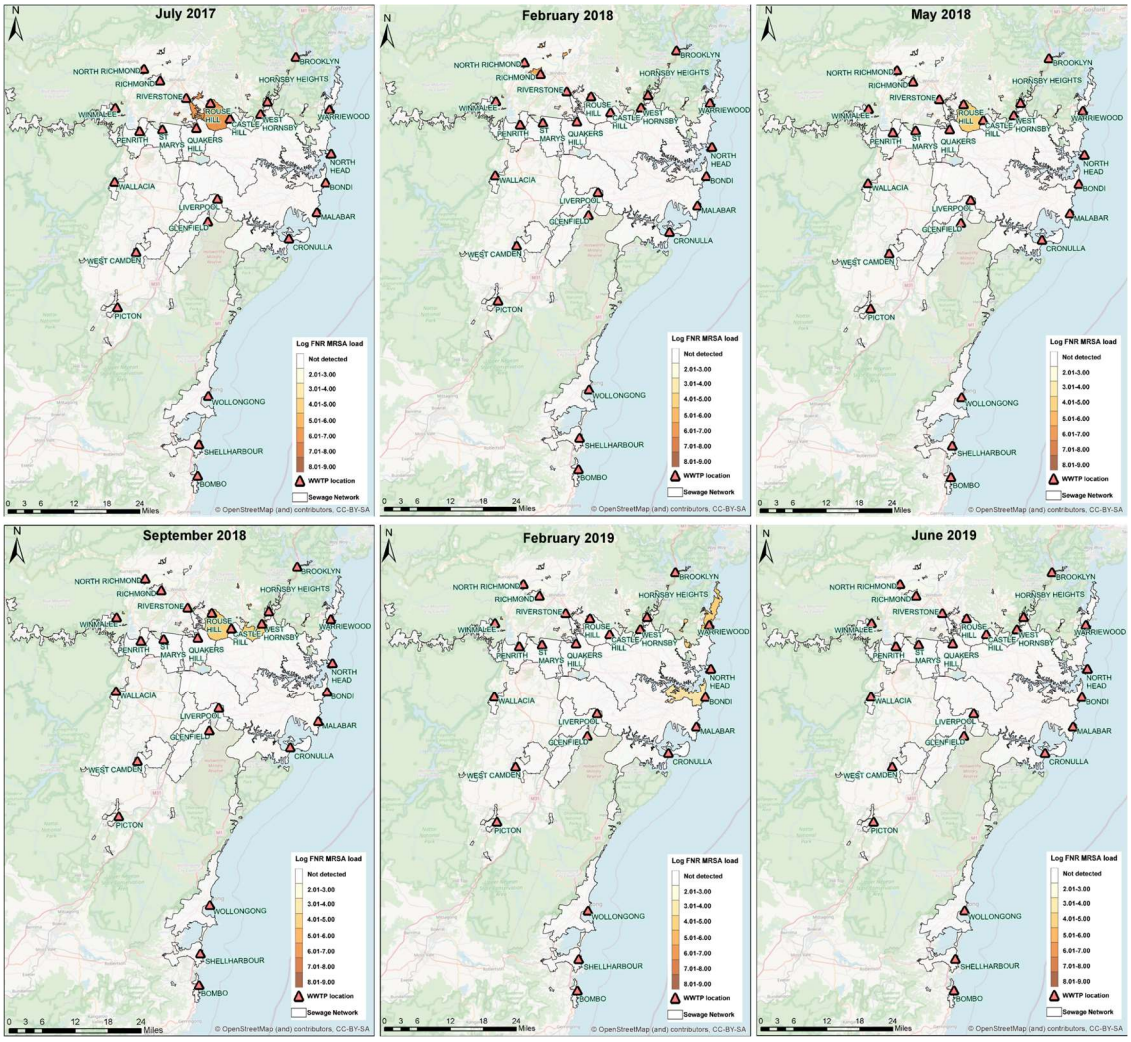


Figure 2.5 Choropleth maps showing the spatial and temporal distribution of the flow normalised relative (FNR) load of methicillin-resistant *Staphylococcus aureus* (MRSA) across 25 WWTP catchments in Sydney.

2.3.2 Factors associated with the FNR load of AMR isolates

The correlation analysis identified several factors significantly associated with the FNR ESBL-E load (Table S2.2). Univariate regression analysis showed that the FNR ESBL-E load was positively correlated with the proportion of the population who had completed vocational education, the number of schools per 10,000 population, the number of small public hospitals per 10,000 population, the average length of hospital stay in the last three months, the number of elective surgeries in the last three months, the number of all RACFs per 10,000 population, the number of large-sized RACFs per 10,000 population, the number of medium-sized RACFs in per 10,000 population, and the number of GPs per 10,000 population (Table 2.1). In contrast, a negative correlation of the FNR ESBL-

E load was found with the population density, the proportion of the population between 19 to 50 years of age, and the proportion of the population who completed at least high school (Table 2.1). The final multiple regression analysis followed by stepwise model selection and all subset regression analysis (Figure S2.2) identified a significant negative association between the FNR ESBL-E load and the number of medium-sized RACFs per 10,000 population ($\beta = -2.01$, $p = 0.01$), and a significant positive association between the FNR ESBL-E load and the proportion of the population between 19 to 50 years of age ($\beta = 0.07$, $p = 0.013$), the proportion of the population who had completed vocational education ($\beta = 0.13$, $p < 0.001$), and the average length of hospital stay in the last three months ($\beta = 1.48$, $p = 0.024$) (Table 2.1). The final selected model had an adjusted R^2 value of 0.36.

The FNR CRE load was significantly correlated with several factors (Table S2.3). Univariate regression analysis identified a direct association between the FNR CRE load and the number of co-located large hospitals per 10,000 population, the number of small public hospitals per 10,000 population, and the average length of hospital stay in the last three months (Table 2.2). An inverse association was observed with the number of all hospitals per 10,000 population (Table 2.2). Multiple linear regression analysis identified a single significant association between the FNR CRE load and the average length of hospital stay in the last three months (adjusted $R^2 = 0.40$, $\beta = 5.78$, $p = 0.005$) (Table 2.2, Figure S2.3).

Significant correlations were identified between the FNR VRE load and several factors (Table S2.4). Univariate regression analysis identified a negative relationship between VRE abundance and the population density, the proportion of the population between 19 to 50 years of age, and the proportion of the population who had completed at least high school (Table 2.3). Conversely, a positive correlation was found with the proportion of the population who had completed vocational education, the number of schools per 10,000 population and the number of co-located large hospitals per 10,000 population (Table 2.3). The final multiple regression analysis followed by stepwise model selection and all subset regression analyses (Figure S2.4) identified a significant association for VRE abundance with the number of schools per 10,000 population ($\beta = 0.29$, $p < 0.001$) (Table 2.3). This final model had an adjusted $R^2 = 0.42$.

Table 2.1 Univariate and multiple linear regression analysis using log_{10} flow normalised relative (FNR) load of extended-spectrum β-lactamases-producing *Enterobacteriaceae* (ESBL-E) in wastewater as the outcome.

Variables	Univariate regression					Multiple regression Model ($R^2_{multiple}$ = 0.41; $R^2_{adjusted}$ = 0.36)			
	β coefficient	95% confidence interval	Std. Error	*p* value	$R^2_{multiple}$	β coefficient	95% confidence interval	Std. Error	*p* value
Log_{10} population density per square kilometre[†]	-1.65	-2.13, -1.18	0.24	<0.001	0.24				
Proportion of the population who had completed at least high school[†]	-0.03	-0.05, -0.01	0.01	0.001	0.07				
Proportion of the population who had completed vocational education[#]	0.08	0.05, 0.11	0.02	<0.001	0.13	0.13	0.06, 0.20	0.03	<0.001
Proportion of the population between 19 to 50 years of age[#]	-0.06	-0.08, -0.04	0.01	<0.001	0.16	0.07	0.01, 0.12	0.02	0.013
Number of schools per 10,000 population[#]	0.20	0.15, 0.26	0.03	<0.001	0.28				
Square root of the number of small public hospitals per 10,000 population[§]	2.08	0.97, 3.18	0.55	<0.001	0.24				
Average length of hospital stay in the last three months per 10,000 population[#]	1.87	0.98, 2.77	0.45	<0.001	0.21	1.48	0.20, 2.76	0.64	0.024
Log_{10} number of elective surgeries in the last three months per 10,000 population[#]	0.66	0.16, 1.16	0.15	0.01	0.08				
Number of all RACFs per 10,000 population[#]	0.45	0.30, 0.61	0.08	<0.001	0.23	0.55	-0.12, 1.22	0.33	0.11
Number of large-sized RACFs per 10,000 population [‡]	0.60	0.36, 0.84	0.12	<0.001	0.19				
Number of medium-sized RACFs per 10,000 population[#]	0.64	0.42, 0.85	0.10	<0.001	0.26	-2.01	-3.52, -0.49	0.75	0.01
Log_{10} number of GPs per 10,000 population[#]	2.12	1.38, 2.86	0.37	<0.001	0.18				

[#] Included in the multiple regression analysis.
[†] Excluded from multiple regression due to multicollinearity (VIF > 10).
[§] Excluded from multiple regression due to a large number of missing values.
[‡] Excluded from multiple regression due to a strong correlation with the ratio of all RACFs (r = 0.87).

Table 2.2 Univariate and multiple linear regression analysis using log_{10} flow normalised relative (FNR) load of carbapenem-resistant *Enterobacteriaceae* (CRE) in wastewater as the outcome.

Variables	Univariate regression					Multiple regression Model ($R^2_{multiple}$ = 0.44; $R^2_{adjusted}$ = 0.40)			
	β coefficient	95% confidence interval	Std. Error	*p* value	$R^2_{multiple}$	β coefficient	95% confidence interval	Std. Error	*p* value
Proportion of the population between 19 to 50 years of age*	-0.02	-0.06, 0.03	0.02	0.53	0.02				
Number of all hospitals per 10,000 population[#]	-1.09	-4.08, -0.06	1.43	0.03	0.13				
Square root of the number of small public hospitals per 10,000 population[§†]	13.68	3.22, 24.13	4.53	0.02	0.53				
Average length of hospital stay in the last three months per 10,000 population[#]	5.78	2.01, 9.55	1.76	0.005	0.44	5.78	2.01, 9.55	1.76	0.005
Number of co-located large hospitals per 10,000 population[§]	21.72	3.44, 39.99	7.46	0.03	0.58				

[#] Included in the multiple regression analysis.
[†] Excluded from multiple regression due to multicollinearity (VIF > 10).
[§] Excluded from multiple regression due to a large number of missing values.
[*] Excluded from multiple regression due to non-significant *p* value in univariate analysis.

Table 2.3 Univariate and multiple linear regression analysis using log_{10} flow normalised relative (FNR) load of vancomycin-resistant enterococci (VRE) in wastewater as the outcome.

Variables	Univariate regression					Multiple regression Model ($R^2_{multiple}$ = 0.45; $R^2_{adjusted}$ = 0.42)			
	β coefficient	95% confidence interval	Std. Error	*p* value	$R^2_{multiple}$	β coefficient	95% confidence interval	Std. Error	*p* value
Log_{10} population density per square kilometre[#]	-2.06	-3.31, -0.81	0.61	0.002	0.23				
Proportion of the population between 19 to 50 years of age[#]	-0.08	-0.13, -0.03	0.03	0.002	0.23				
Proportion of the population who had completed at least high school[#]	-0.04	-0.10, -0.01	0.02	0.04	0.90				
Proportion of the population who had completed vocational education[#]	0.11	0.03, 0.19	0.04	0.008	0.18	0.07	-0.00, 0.14	0.03	0.054
Number of schools per 10,000 population[#]	0.33	0.19, 0.46	0.07	<0.001	0.39	0.29	0.15, 0.43	0.07	<0.001
Number of co-located large hospitals per 10,000 population[§]	85.3	-17.57, 188.18	32.32	0.05	0.70				

[#] Included in the multiple regression analysis.
[§] Excluded from multiple regression due to a large number of missing values.

2.4 Discussion

I investigated whether regular monitoring of wastewater could establish a baseline of a selection of clinically important AMR bacteria in the Sydney general community and whether the AMR pathogen load in wastewater could be explained by accessible demographic, socioeconomic and healthcare infection-related parameters. By repeatedly testing wastewater from 25 WWTPs that service 5.2 million population from 1.8 million households across the Greater Sydney area, I was able to monitor the geospatial-temporal trend of four clinically significant human pathogens over a three-year period and explain the observed variation of ESBL-E, CRE, and VRE bacterial load in wastewater.

My results demonstrate stability for the total bacterial load owing to the variation being less than one log standard deviation across all the sampling sites and periods. ESBL-E was the only persistent AMR bacteria in each sample period, but at varying amounts across all 25 sites. My study provides a better representation of ESBL-E in the community than previous studies in Olsztyn, Poland (Korzeniewska & Harnisz, 2013) and Oslo, Norway (Paulshus et al., 2019), which also reported persistent ESBL-E isolates with repeated sampling, but only from a single WWTP representing one wastewater catchment. As *Enterobacteriaceae* belong to the natural microbiota of the human intestinal tract and represent the majority of bacterial communities in wastewater (Filipkowska, 2003; Korzeniewska et al., 2009), the widespread occurrence of these bacterial populations in wastewater was not surprising. The widespread occurrence of ESBL-E has also been reported in the broader community sampled from multiple sources such as the environment, food and companion animals, travellers, and various households (Doi et al., 2017). The persistent presence of ESBL-E in wastewater from the Greater Sydney region suggests that once the pathogen is acquired, there is an enduring endemicity in the sampled communities.

The FNR ESBL-E load in wastewater was weakly associated with two demographic parameters, vocational education and age, and two healthcare infection-related factors, the average length of hospital stay and medium-sized RACFs, that explained only a third of the variance observed in the data ($R^2 = 0.36$). Several clinical and community-based studies have indicated that the older population is more likely to have acquired ESBL-E infections than the younger population (Colodner et al., 2004; Ben-Ami et al., 2009;

Friedmann et al., 2009). In contrast, my study found a positive association with a younger population, 19 to 50 years of age. This result may indicate a shift in ESBL-E epidemiology in the community, driven in part by a younger population. Previous studies have also found a positive and consistent effect of higher education on improved health (Ross & Wu, 1995; Mirowsky & Ross, 2003). Similar relationships between ESBL-E, vocational education and age may be a proxy for each other, although no collinearity was identified. My finding of the positive association of ESBL-E with the average length of hospital stay in the last three months is consistent with clinical studies (Bisson et al., 2002; Friedmann et al., 2009). The population in RACFs has also previously been shown to have a higher risk of getting ESBL-E infections (Rooney et al., 2009; Stuart et al., 2011; Lim et al., 2014). However, my finding suggests that at an aggregated level for medium-sized RACFs, this was not always true. Overall, these results suggest that the observed ESBL-E profile in wastewater may have been driven by small contributions of demographic and healthcare infection-related factors (and other unidentified factors). However, for the objective of monitoring wastewater for a change in geospatial or temporal trends in ESBL-E, I identified a consistent and persistent level of ESBL-E across the residents of Greater Sydney.

The CRE, VRE, and MRSA isolates were occasionally detected at different WWTPs and sampling periods. The lack of detection of CRE, VRE, and MRSA does not necessarily mean absence, but rather that the levels may have been below the detection limit used in this study. Despite this, my results showed strong temporal and spatial dynamics for these isolates. Direct comparisons of the observed trends in resistant bacteria in wastewater with trends in healthcare settings were not possible due to the absence of clinical data specifically for the Greater Sydney region at the catchment level as the reported AMR aggregated across healthcare facilities (AGAR, 2019; CARAlert, 2019; AURA, 2021). Yet, for CRE loads, the absence of a seasonal pattern over the sampling periods was consistent with healthcare-based aggregated surveillance findings (CARAlert, 2019).

While several healthcare infection-related factors were correlated with the FNR CRE load, the average length of hospital stays in the last three months explained less than half (adjusted $R^2 = 0.40$) of the variation in wastewater. My result is consistent with a recent case-control study by Lin et al. (2019), which developed a model of CRE carriage using

a hospital discharge database and predicted the mean length of stay in both short-term and long-term acute care hospitals as an important risk factor for CRE. A reduction in the average length of hospital stays also augmented the effort to control the spread of CRE, such as *K. pneumoniae*, in 14 hospitals in the USA (Landman et al., 2012). An extended period of hospital stay may be a proxy for the acquisition of CRE from the selection pressure of prolonged exposure to broad-spectrum antibiotics, such as extended-spectrum penicillins, cephalosporins, fluoroquinolones, and carbapenems (Jeon et al., 2008; Wu et al., 2011; Teo et al., 2012; Holmes et al., 2016). Transmission of CRE isolates can also occur in the absence of adequate infection control measures, such as isolation precautions, contamination of the hospital environment, especially handwashing resources, and poor hand hygiene compliance by healthcare workers (Munoz-Price et al., 2010; CDC, 2012; Schechner et al., 2013; Leitner et al., 2015; Rossini et al., 2016; WHO, 2019a). Additionally, I evaluated whether the hospitals within each wastewater catchment have biased the association of the FNR CRE load towards the duration of hospital stay. I estimated that if all hospital beds within each catchment were occupied by patients carrying AMR pathogens during the time of sampling, it only represents around 0.05 to 0.51% of the population across all wastewater catchments. So, the hospitals within a catchment area are unlikely to influence the association between CRE load and the average length of hospital stay. Therefore, my finding is likely the result of hospital acquisition and then spreads into the community once patients are discharged from the hospital. My monitoring does not provide detailed clinical observations, but it does provide a baseline of the presence and trends of CRE in the Greater Sydney region. The association of CRE and ESBL-E loads, as mentioned earlier, with the average length of hospital stay may provide the leverage needed to safely reduce or examine those preventable practices during extended stays that will increase the acquisition of these AMR isolates.

Surprisingly, the FNR VRE load was not significantly correlated with any hospital or healthcare infection-related factors in the multiple regression analysis. VRE isolates are usually reported from the healthcare settings and cause a wide range of illnesses, including surgical site, urinary tract, and bloodstream infections (CDC, 2013; Sengupta et al., 2013; AURA, 2019). VRE is an infection experienced mostly by the elderly or immunocompromised (AURA, 2019), but my regression analysis identified that the FNR

VRE load was driven by a hard-to-interpret demographic parameter (i.e. the number of schools per 10,000 population). This variable, however, explained almost half (adjusted $R^2 = 0.42$) of the variance, but it may be a proxy for a variable yet identified. Interestingly, neither RACFs nor any healthcare infection-related factors were associated with VRE. Surprisingly, a detectable VRE faecal carriage rate of 0.8% was observed in primary school children in İstanbul, Turkey (Çakırlar et al., 2019). The weak but statistically significant association of the VRE load with the ratio of schools to population may warrant furthermore direct sampling of school wastewater.

This study may have some limitations and biases. First, the use of grab samples may not always be representative of the total population within a wastewater catchment, as the bacterial load may vary with the time of sampling. However, I found no statistical support for an association of the bacterial load with the sampling time, and I also normalised the resistant bacterial load with the total bacterial load. Second, confirmation of CRE based on resistance to meropenem using VITEK 2 AES may not be able to detect OXA-48-type carbapenemase, many of which were susceptible to meropenem (Oueslati et al., 2015). This may lead to an underestimation of the CRE isolates present in the community. Third, quantification based on the confirmation of one representative colony for each characteristic colour type may limit the accuracy of the counts. However, it was not practical to confirm the identity of every single colony based on mass spectrometry or VITEK 2 analysis. Fourth, culture-based methods are limited in detecting non-culturable or slow-growing bacteria, which may be present in the wastewater but not captured by the culture media used. Fifth, culture-based methods do not detect antibiotic resistance genes, which may be present in bacteria but not expressed (Karkman et al., 2018). Consequently, the overall resistance profile in the community may not be accurately reflected. Therefore, it is recommended to use a combination of culture-based and molecular methods (such as qPCR/ PCR/ metagenomics) for a more comprehensive and accurate assessment of antibiotic-resistant bacteria in wastewater. However, culture-independent molecular methods are limited in identifying novel and emerging bacterial strains in the community (see Section 5.3 on pages 127-128 of Chapter 5 for more details).

Nevertheless, the use of wastewater testing to monitor AMR in the general Sydney community without an invasion of privacy provided a baseline of clinically relevant

bacterial abundance for ESBL-E, CRE, VRE, and MRSA. Additionally, I found that the flow normalised relative load of resistant bacterial isolates was not strongly influenced by any hospital or healthcare infection-related variables, except for CRE. In particular, the FNR CRE load was associated with the average length of hospital stay in the last three months. With accessible community samples, wastewater-based monitoring can be used for early warning alerts of an unexpected rise in resistance, such as during outbreaks or epidemics. For instance, wastewater-based monitoring of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has recently been adopted as an early warning system for the prevalence of both symptomatic and asymptomatic infections in the community located within a WWTP catchment (Ahmed et al., 2020; Gonzalez et al., 2020; La Rosa et al., 2020; Wu et al., 2020). While current hospital or healthcare infection surveillance is essential to capture AMR for individual patients, my wastewater-based monitoring provides a simple, reliable and affordable method to assess changes in abundance, location, and trends in AMR at the non-hospitalised community level.

2.5 Conclusion

- The total bacterial loads varied with less than one log standard deviation across all sampling sites and periods tested, indicating relative stability.
- ESBL-E isolates were consistently found in all wastewater catchments during the three years of sampling at endemic levels in the Sydney region.
- CRE, VRE, and MRSA isolates were occasionally detected at different sampling sites and periods. Geospatial-temporal dynamics were observed for these bacterial loads without any clear temporal patterns.
- The FNR ESBL-E load was associated with both demographic and healthcare infection-related factors (adjusted $R^2 = 0.36$, $p < 0.001$), and the FNR VRE load was influenced by the demographic variable (adjusted $R^2 = 0.42$, $p < 0.001$). While the FNR CRE load was driven mostly by the healthcare infection-related parameter (adjusted $R^2 = 0.40$, $p = 0.005$).
- Along with healthcare infection surveillance, wastewater-based surveillance could be used to monitor the geospatial and temporal trends of AMR in the community that can assist in management strategies to mitigate the emergence and spread of AMR.

Chapter 3 Genomic characterisation of extended-spectrum β-lactamases-producing and carbapenem-resistant *E. coli* from urban wastewater indicates their emergence and dissemination beyond clinical settings

3.1 Introduction

Escherichia coli is a versatile bacterial species in the family *Enterobacteriaceae* and comprises strains that are harmless inhabitants, as well as pathogenic variants capable of causing intestinal or extraintestinal infections in humans and animals (Johnson & Russo, 2002; Kaper et al., 2004; Croxen et al., 2013; Sarowska et al., 2019). *E. coli* is subdivided into different pathotypes based on its infected host, site of infections, types of acquired virulence factors, and clinical manifestation of diseases (Kaper et al., 2004; Croxen & Finlay, 2010). The *E. coli* pathotypes associated with enteric infections are most prevalent in developing countries and include adherent invasive *E. coli* (AIEC), diffusely adherent *E. coli* (DAEC), enteroaggregative *E. coli* (EAEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), and enterotoxigenic *E. coli* (ETEC) (Kaper et al., 2004; Croxen et al., 2013). Pathotypes related to extraintestinal infections comprise uropathogenic *E. coli* (UPEC), septicaemia-associated *E. coli* (SEPEC), neonatal meningitis-causing *E. coli* (NMEC), and avian pathogenic *E. coli* (APEC) (Croxen & Finlay, 2010; Sarowska et al., 2019). While treatment of human enteric infections is usually supportive and targeted at maintaining hydration and electrolyte balance, extraintestinal infections often require treatment with antibiotics, such as β-lactams, aminoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole (Pitout, 2012; Croxen et al., 2013). However, *E. coli* strains that are resistant to multiple antibiotic classes, including last resort carbapenems and polymyxins, are increasing worldwide (WHO, 2014), leaving fewer or sometimes no effective antibiotics for treatment. This may cause increased hospitalisation rates, morbidity, mortality, and treatment costs (Magiorakos et al., 2012; Bhatt et al., 2015; Iredell et al., 2016; Batalla-Bocaling et al., 2021).

The most common resistance mechanism in *E. coli* is the production of different β-lactamases that render these organisms resistant to clinically important β-lactam

antibiotics such as penicillins, cephalosporins, and carbapenems (Pitout & Laupland, 2008; Bush & Bradford, 2019; Nordmann & Poirel, 2019; Peirano & Pitout, 2019). Among the different β-lactamases produced by *E. coli*, ESBLs, AmpC β-lactamases, and carbapenemases remain the most significant from a clinical point of view owing to their increasing prevalence around the globe as a cause of antibiotic-resistant infections (Pitout & Laupland, 2008; Peirano & Pitout, 2019; Meini et al., 2019). ESBL confer resistance to penicillins, first-, second-, and third-generation cephalosporins (i.e. cefazolin, cefuroxime, ceftriaxone, and ceftazidime), some fourth-generation cephalosporins (i.e. cefepime), and monobactams (i.e. aztreonam), but cannot hydrolyse cephamycins (i.e. cefoxitin and cefotetan) and carbapenems (i.e. meropenem, imipenem, and ertapenem), and are inhibited by β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam (Pitout & Laupland, 2008; Peirano & Pitout, 2019). The majority of the ESBLs in *E. coli* belong to Ambler class A (classified based on amino acid sequence homology) and include the sulfhydryl variable (SHV)-, temoniera (TEM)- and cefotaximase-Munich (CTX-M)-types (Paterson & Bonomo, 2005; Bush & Jacoby, 2010; Bush, 2020), that are often carried by mobile genetic elements (MGEs) and contribute to their mobilisation within and between bacterial hosts via horizontal gene transfer (Peirano & Pitout, 2019; Bush, 2020). Chromosomally encoded class C β-lactamases such as AmpC in *E. coli* can hydrolyse penicillins, cephalosporins, cephamycins, and monobactams when overproduced mainly due to upregulation of the *ampC* gene because of mutations in the promoter/attenuator region (Nelson & Elisha, 1999; Caroff et al., 2000; Tracz et al., 2005; Peter-Getzlaff et al., 2011). AmpC-overproducing *E. coli* isolates are usually susceptible to carbapenems and are not inhibited by β-lactamase inhibitors clavulanic acid, sulbactam, and tazobactam (Jacoby, 2009; Meini et al., 2019). In addition, *E. coli* can acquire plasmid-encoded AmpCs (i.e. cephamycinase [CMY]-type) that are often expressed at high levels due to strong promoters and high copy numbers and, therefore, can confer a similar level of β-lactam resistance (Jacoby, 2009; Meini et al., 2019). In *E. coli*, the acquisition of carbapenemase genes is the most important resistance mechanism against last resort carbapenems that also confer resistance to other β-lactam antibiotics such as penicillins, cephalosporins and monobactams, and are poorly inhibited by β-lactamase inhibitors clavulanic acid, sulbactam and tazobactam (Queenan & Bush, 2007; Nordmann & Poirel, 2019; Tooke et al., 2019). The carbapenemases primarily reported in *E. coli* belong to Ambler class A *Klebsiella pneumoniae*

carbapenemases (KPC)-type, class B metallo-β-lactamases (MBL) including imipenemase (IMP)-, Verona integron-encoded metallo-β-lactamases (VIM)-, and New Delhi metallo-β-lactamase (NDM)-types, or class D carbapenem-hydrolysing oxacillinase (OXA) such as OXA-48-types (Nordmann et al., 2011; Poirel et al., 2012; Nordmann & Poirel, 2014; Boyd et al., 2020). In addition, carbapenem resistance in *E. coli* is also attributed to the deletion or reduction of outer membrane porin (OMP) in the presence/absence of production of ESBLs and/or AmpC β-lactamases (Nordmann et al., 2012). Other clinically significant resistance mechanisms in *E. coli* include the production of aminoglycoside-modifying enzymes and ribosomal methyltransferases that confer resistance to aminoglycosides; quinolone resistance-conferring mutations at target sites, such as topoisomerases; and the drug-resistant variant of the chromosomal target enzymes dihydropteroate synthase and dihydrofolate reductase conferring resistance to trimethoprim-sulfamethoxazole (Sköld, 2001; Hooper & Jacoby, 2016; Poey et al., 2019; Wachino et al., 2020).

Of particular concern is the acquisition of plasmids carrying multiple resistance determinants, allowing the emergence and rapid local as well as global dissemination of multidrug-resistant *E. coli* (Carattoli, 2013; Pilla & Tang, 2018; Rozwandowicz et al., 2018). The global dissemination of multidrug-resistant *E. coli* is also mediated by a small number of epidemiologically successful clones (Woodford et al., 2011; Mathers et al., 2015). Among these, the sequence type 131 (ST131) is the most prevalent high-risk clone owing to the worldwide distribution, high level of virulence and fitness, persistence and ease of transmission between human and non-human hosts, and resistance to β-lactams such as third-generation cephalosporins and fluoroquinolones (Petty et al., 2014; Decano & Downing, 2019; Pitout et al., 2020). Examples of other clinically significant multidrug-resistant high-risk *E. coli* clones include ST10, ST38, ST69, ST73, ST95, ST155, ST393, ST405, and ST648 (Riley, 2014; Manges et al., 2019). Several recent reports also documented carbapenemase-producing ST167, ST410, and fluoroquinolone-resistant ST1193 as emerging international high-risk clones (Johnson et al., 2018; Roer et al., 2018; Tchesnokova et al., 2018; Garcia-Fernandez et al., 2020).

In Australia, *E. coli* has been considered a priority pathogen of clinical significance and resistance to common agents used to treat *E. coli* infections has been monitored at the

national level using the healthcare-based Antimicrobial Use and Resistance in Australia (AURA) surveillance system (AURA, 2016). Resistance of *E. coli* isolated from healthcare- and community-acquired infections to essential antibiotics such as third-generation cephalosporins, fluoroquinolones, and aminoglycosides has been reported to be increasing in recent years in Australia (AURA, 2021). *E. coli* resistance to last resort antibiotics such as carbapenems remains rare in Australia, based on isolates from blood and urine culture (less than 0.1% of *E. coli* in 2019) (AURA, 2021).

A major limitation of the AURA and other international healthcare infection surveillance is the inability to monitor the emergence and spread of resistance in *E. coli* beyond clinical settings, that is, in the community and non-human sources, such as animals and the environment. These non-human and community-based sources have been proposed as potential reservoirs for amplifying and transmitting resistant organisms or genes across different niches (Bailey et al., 2010; Bélanger et al., 2013; Szmolka & Nagy, 2013; Woolhouse et al., 2015). Therefore, it is crucial to monitor antibiotic-resistant *E. coli* beyond the clinal setting to effectively track and control the emergence, spread, and transmission of the ever-increasing rate of resistance. As wastewater receives bacteria from humans, animals, and environmental sources, routine wastewater-based surveillance could be used to monitor the occurrence and diversity of antibiotic-resistant *E. coli* that might be circulating in the community (Kühn et al., 2003; Reinthaler et al., 2013; Kwak et al., 2015).

In Chapter 2, I used wastewater-based surveillance as a monitoring tool for antibiotic resistance trends in several priority pathogens in the Sydney community. From untreated influent wastewater collected at six-time points between 2017 and 2019 from 25 Sydney wastewater treatment plants (WWTPs), ESBL-producing *E. coli* (ESBL-EC) were consistently, and carbapenem-resistant *E. coli* (CR-EC) were sporadically detected. Here, I aimed to understand what antibiotic resistance patterns these wastewater *E. coli* isolates have, whether these isolates are phylogenetically related to any known pandemic high-risk clones, what molecular mechanisms underpin their resistance, as well as their potential for virulence. I also aimed to identify the potential of these isolates to acquire and transfer antibiotic resistance genes and virulence determinants.

3.2 Methods

3.2.1 Bacterial isolation, identification, and antibiotic susceptibility testing

Isolates exhibiting resistance against the largest number of antibiotics that are critically important for human health (WHO, 2019b) were selected (n = 8) for this study from a collection of CR-EC and ESBL-EC isolates (n = 163). This collection was derived from untreated influent wastewater during the wastewater-based surveillance in Sydney, New South Wales, Australia, from 2017 to 2019, using methods described in Chapter 2.

The antibiotic susceptibility of *E. coli* isolates against 18 different antibiotics contained in the AST-N246 card was determined using a VITEK 2 instrument (bioMérieux, Vitek-Australia Pty Ltd) following the manufacturer's instruction and the Clinical and Laboratory Standards Institute's guidelines (CLSI, 2018), as described in Chapter 2. Resistance to fosfomycin, not included in the VITEK2 AST-N246 card, was determined by the Kirby-Bauer disk diffusion method (Bauer et al., 1966) using 200µg fosfomycin plus 50µg glucose-6-phosphate disk (Mast Group Ltd., Bootle, UK) according to the CLSI guidelines (CLSI, 2018).

3.2.2 Whole-genome sequencing, assembly, and annotation

Selected *E. coli* strains (n = 8) were revived from glycerol stocks on horse blood agar (HBA) plates (bioMérieux), and a single colony was picked and grown in LB broth at 37°C with constant agitation at a speed of 120 rpm. Genomic DNA was extracted from the overnight culture using the Monarch® Genomic DNA Purification Kit (New England Biolabs, Australia), following the manufacturer's instructions. Sequencing libraries were prepared with the Nextera XT kit, and 250 base pair (bp) paired-end sequencing was performed on the Illumina MiSeq platform at the Ramaciotti Centre for Genomics at the UNSW Sydney. The quality of raw sequencing reads was assessed using FastQC v.0.11.8 (Andrews, 2010). Adapters and other Illumina-specific sequences, bases with a Phred quality score below 25 at the start and end of a read, and reads with a minimum length of 50 bases were trimmed with Trimmomatic v.0.38 (Bolger et al., 2014). *De novo* assembly of quality-filtered reads was performed using the default parameters of the SPAdes genome assembler v.3.15.0 (Bankevich et al., 2012). The quality of the assembled

genomes was evaluated with Quast v.5.0.2 (Gurevich et al., 2013), and the genomes were annotated using Prokka v.1.14.5 with the default databases (Seemann, 2014).

3.2.3 Bioinformatic and phylogenetic analysis

The sequence types (ST) of assembled *E. coli* genomes were predicted using the MLST 2.0 tool (Larsen et al., 2012) based on Achtman's multilocus sequence typing (MLST) scheme (Wirth et al., 2006). In addition, the antibiotic resistance genes, plasmid replicons and virulence genes were predicted from the genomes using ResFinder 4.0 (Bortolaia et al., 2020), PlasmidFinder 2.1 (Carattoli et al., 2014), and VirulenceFinder 2.0 (Joensen et al., 2014), respectively, using a minimum nucleotide identity of 90% and a minimum alignment coverage of 60%. Contigs containing plasmid replicons were manually inspected for the presence of antibiotic resistance and virulence genes using the ResFinder and VirulenceFinder output, respectively. Antibiotic resistance or virulence gene are considered plasmid-borne if located on a contig with plasmid replicons (Carattoli et al., 2014; Joensen et al., 2014).

The maximum likelihood phylogeny of the *E. coli* genomes was inferred from an alignment of concatenated MLST genes (*adk–fumC–gyrB–icd–mdh–purA–recA*) produced using MAFFT v.7.407 (Katoh & Standley, 2013) with default parameters and a maximum likelihood phylogenetic tree was built applying the general time reversible (GTR)-Gamma model with 1,000 bootstraps in RAxML v.8.2.10 (Stamatakis et al., 2014). To contextualise the broader relevance of wastewater isolates, a total of 1,036 *E. coli* genomes belonging to similar ST complexes from around the world were randomly retrieved from the Enterobase database (Zhou et al., 2020), annotated using Prokka and antibiotic resistance genes were predicted using ResFinder. Comparative analysis of 248,388 strains available in Enterobase (https://enterobase.warwick.ac.uk/species/index/ecoli [accessed, 23/02/2023]) in terms of phylogenetic analysis and gene comparison was not practical. Therefore, a random sub-selection was performed to avoid bias. The pan-genome of the study and global isolates were estimated for each of the ST complexes using Roary v.3.12.0 (Page et al., 2015) with default parameters. Maximum likelihood phylogenetic trees were constructed using RAxML with the GTR-Gamma model from the alignments of concatenated core coding sequence (present in 99% of isolates) created

using the -mafft option in Roary. Phylogenetic trees were visualised with metadata in iTOL v.6.3 (Letunic & Bork, 2021).

Mutations were predicted from the read alignment of the study isolates with the closely related reference genome for each ST obtained from the National Center for Biotechnology Information (NCBI) reference sequence (RefSeq) database (O'Leary et al., 2016) using Breseq v.0.35.4 (Deatherage & Barrick, 2014) with default parameters. Before Breseq analysis, the genomic relatedness of the study and the reference genomes was verified by estimating the average nucleotide identity of the shared coding regions using FastANI v.1.33 (Jain et al., 2018). The predicted mutations in the selected genes of the study isolates were verified by aligning their respective nucleotide and amino acid sequences with the reference sequences using clustal omega (Sievers & Higgins, 2014). The impact of amino acid substitutions (neutral or deleterious) on the biological function of proteins encoded by selected genes was predicted using the protein variation effect analyser v.1.1.3 (PROVEAN) tool (Choi & Chan, 2015).

3.3 Results and discussion

3.3.1 Antibiotic susceptibility pattern

To understand whether wastewater *E. coli* isolates have unique or unusual antibiotic resistance patterns, I assessed 12 CR-EC and 151 ESBL-EC isolates for resistance against 19 common antibiotics (Supplementary Table S3.1). The WHO (WHO, 2019b) categorises antibiotic classes as 'critically important' for human medicine when these are the sole therapy or one of the few alternatives available to treat serious bacterial infections (criterion 1) and when bacterial infections may be transmitted from non-human sources or acquire resistance genes from non-human sources (criterion 2). The 'highly important' and 'important' antibiotic classes are those that meet one of the two criteria and none of the criteria, respectively (WHO, 2019b). In addition, antibiotics within the critically important classes are prioritised based on three factors to guide the urgent allocation of resources for the risk management of resistance (WHO, 2019b). The first prioritisation factor relates to the large number of people who might require an antibiotic, for which there are very few alternatives available. The second prioritisation factor relates to the high-frequency of usage of that antibiotic in human medicine, and the third prioritisation

factor relates to extensive evidence on the transmission of resistant bacteria or genes against that antibiotic from non-human sources. Critically important antibiotics are classified as the highest priority if all three prioritisation factors are met and high priority if not all the factors are met (WHO, 2019b).

Among the resistance patterns observed, some CR-EC isolates exhibited high-level resistance against almost all antibiotics tested (Table S3.1), which is rare in Australia based on data from human clinical isolates (Fasugba et al., 2019; Hastak et al., 2020; AURA, 2021). CR-EC isolates G4, G5, and G8 were resistant to all the highest priority critically important antibiotics such as third- and fourth-generation cephalosporins (i.e. ceftazidime, ceftriaxone, and cefepime) and fluoroquinolones (i.e. ciprofloxacin and norfloxacin) (Table 3.1). In addition, these isolates exhibited resistance to several high priority critically important antibiotics such as third-generation penicillin or aminopenicillin (i.e. ampicillin), carbapenem (i.e. meropenem), penicillins with β-lactamase inhibitors (i.e. amoxicillin/clavulanic acid and piperacillin/tazobactam), and aminoglycosides (i.e. gentamicin, tobramycin, and amikacin). The CR-EC isolates G4, G5, and G8 were sensitive only to the phosphonic acid derivative (i.e. fosfomycin) (Table 3.1). CR-EC isolate G16 also exhibited a similar susceptibility pattern against high priority critically important antibiotic classes but was sensitive to amikacin (Table 3.1). Furthermore, the selected CR-EC isolates showed resistance to all tested highly important antibiotics such as first- and second-generation cephalosporins (i.e. cefazolin and cefoxitin, respectively), dihydrofolate reductase inhibitor (i.e. trimethoprim), and dihydrofolate reductase inhibitor and sulfonamide combinations (i.e. trimethoprim-sulfamethoxazole) (Table 3.1). Resistance towards important antibiotics such as the nitrofurantoin derivative (i.e. nitrofurantoin) was only exhibited by isolate G8, whereas the G4, G5, and G16 isolates showed intermediate susceptibility (Table 3.1). All CR-EC isolates were resistant to the uncategorised ticarcillin/clavulanic acid (Table 3.1). CR-EC isolates G4, G5, G8, and G16 with their resistance to all but one or two of the tested antibiotic classes can be considered extensively drug-resistant (XDR) (Magiorakos et al., 2012), leaving only intravenous fosfomycin as the last resort therapy. Infections caused by XDR *E. coli* are extremely challenging to treat, resulting in prolonged hospitalisation, increased treatment costs, and poor patient outcomes, which is alarming (Magiorakos et al., 2012; Karaiskos and Giamarellou, 2014; Bhatt et al., 2015; Batalla-Bocaling et al.,

2021). Therefore, a comprehensive understanding of resistance mechanisms and the identification of possible transmission sources of resistant bacteria and their genes are essential to support appropriate treatment regimens and interventions to control the emergence and spread of such XDR CR-EC isolates.

Among the diverse resistance patterns observed for ESBL-EC (Table S3.1), I chose four isolates exhibiting resistance against the highest number of critically important and highly important antibiotics for human health for further analysis (WHO, 2019b) (Table 3.1). Specifically, ESBL-EC isolates G10, G11, G15, and G17 were multidrug-resistant (MDR) as they were resistant to at least three classes of antibiotics (Magiorakos et al., 2012). These isolates also exhibited resistance towards all tested β-lactam antibiotics except carbapenems (Table 3.1). For instance, resistance was observed towards ceftazidime, ceftriaxone and cefepime (highest priority critically important), ampicillin (high priority critically important), cefoxitin and cefazolin (highly important) (Table 3.1). In addition, these isolates were found to be resistant against nitrofurantoin (important), and susceptible towards norfloxacin (highest priority critically important) and amikacin (high priority critically important). Variable resistant patterns were observed in G10, G11, G15, and G17 isolates against the rest of the tested antibiotics (Table 3.1).

ESBL-EC isolates resistant towards all the commonly prescribed β-lactam antibiotics may limit treatment options and may increase the use of last resort drugs, such as carbapenem, as first-line treatment (Harris et al., 2015). Furthermore, the diverse resistance pattern of ESBL-EC isolates against other tested critically important antibiotics is concerning, as the acquisition of resistance against these classes will further reduce therapeutic options. Therefore, understanding the underlying resistance mechanisms and identifying the possible source of resistant bacteria or genes is essential to track and control the emergence and spread of MDR ESBL-EC bacteria.

Given the information above, CR-EC isolates G4, G5, G8 and G16, and ESBL-EC isolates G10, G11, G15 and G17 were selected and subjected to whole-genome sequencing (WGS) to define their relationship with known *E. coli* lineages, the genetic bases of their resistance as well as virulence, and their potential for acquisition and spread of resistance and virulence-encoding genes.

Table 3.1 Antibiotic susceptibility pattern of the carbapenem-resistant *E. coli* (CR-EC) and extended-spectrum β-lactamases-producing *E. coli* (ESBL-EC) isolated from wastewater.

Isolates	Year of collection	Phenotypes	Antibiotic Classes																		
			β-lactams							Penicillins with β-lactamase inhibitors			Aminoglycosides			Fluoroquinolones		Dihydrofolate reductase inhibitors	Combinations	Nitrofuran derivatives	Phosphonic acid derivatives
			Aminopenicillin	Cephamycin	Cephalosporins				Carbapenem												
			Ampicillin	Cefoxitin	Cefazolin	Ceftazidime	Ceftriaxone	Cefepime	Meropenem	Amoxicillin/clavulanic acid	Piperacillin/tazobactam	Ticarcillin/clavulanic acid	Gentamicin	Tobramycin	Amikacin	Ciprofloxacin	Norfloxacin	Trimethoprim	Trimethoprim-Sulfamethoxazole	Nitrofurantoin	Fosfomycin
G4	2017	CR-EC	**R** (>=32*)	**R** (>=64)	**R** (>=64)	**R** (>=64)	**R** (>=64)	**R** (>=64)	**R** (>=16)	**R** (>=32)	**R** (>=128)	**R** (>=128)	**R** (>=16)	**R** (>=16)	**R** (>=64)	**R** (>=4)	**R** (>=16)	**R** (>=16)	**R** (>=320)	**I** (64)	**S** (27 mm#)
G5	2017	CR-EC	**R** (>=32)	**R** (>=64)	**R** (>=64)	**R** (>=64)	**R** (>=64)	**R** (>=64)	**R** (>=16)	**R** (>=32)	**R** (>=128)	**R** (>=128)	**R** (>=16)	**R** (>=16)	**R** (>=64)	**R** (>=4)	**R** (>=16)	**R** (>=16)	**R** (>=320)	**I** (64)	**S** (27 mm)
G8	2018	CR-EC	**R** (>=32)	**R** (>=64)	**R** (>=64)	**R** (>=64)	**R** (>=64)	**R** (>=64)	**R** (8)	**R** (>=32)	**R** (>=128)	**R** (>=128)	**R** (>=16)	**R** (>=16)	**R** (>=64)	**R** (>=4)	**R** (>=16)	**R** (>=16)	**R** (>=320)	**R** (128)	**S** (23 mm)
G16	2019	CR-EC	**R** (>=32)	**R** (>=64)	**R** (>=64)	**R** (>=64)	**R** (>=64)	**R** (>=64)	**R** (>=16)	**R** (>=32)	**R** (>=128)	**R** (>=128)	**R** (>=16)	**R** (>=16)	**S** (4)	**R** (>=4)	**R** (>=16)	**R** (>=16)	**R** (>=320)	**I** (64)	**S** (26 mm)
G10	2018	ESBL-EC	**R** (>=32)	**R** (32)	**R** (>=64)	**R** (16)	**R** (>=64)	**R** (8)	**S** (<=0.25)	**R** (>=32)	**I** (64)	**S** (16)	**R** (>=16)	**I** (8)	**S** (<=2)	**I** (0.5)	**S** (2)	**S** (<=0.5)	**S** (<=20)	**R** (128)	**S** (21 mm)
G11	2018	ESBL-EC	**R** (>=32)	**R** (>=64)	**R** (>=64)	**R** (>=64)	**R** (>=64)	**R** (>=64)	**S** (<=0.25)	**R** (>=32)	**R** (>=128)	**R** (>=128)	**S** (<=1)	**S** (<=1)	**S** (<=2)	**R** (2)	**S** (4)	**S** (2)	**S** (<=20)	**R** (128)	**S** (21 mm)
G15	2019	ESBL-EC	**R** (>=32)	**R** (32)	**R** (>=64)	**R** (8)	**R** (>=64)	**R** (32)	**S** (<=0.25)	**S** (8)	**S** (16)	**S** (16)	**R** (>=16)	**I** (8)	**S** (<=2)	**S** (<=0.25)	**S** (2)	**R** (>=16)	**R** (>=320)	**R** (256)	**I** (14 mm)
G17	2019	ESBL-EC	**R** (>=32)	**R** (>=64)	**R** (>=64)	**R** (>=64)	**R** (>=64)	**R** (32)	**S** (<=0.25)	**R** (>=32)	**I** (64)	**R** (>=128)	**R** (>=16)	**S** (2)	**S** (<=2)	**R** (2)	**S** (4)	**R** (>=16)	**R** (>=320)	**R** (128)	**S** (28 mm)
Categories of important antibiotics for human medicine (WHO, 2019b)			Critically important (High priority)	Highly important	Highly important	Critically important (Highest priority)	Critically important (Highest priority)	Critically important (Highest priority)	Critically important (High priority)	Critically important (High priority)	Critically important (High priority)	Not available	Critically important (High priority)	Critically important (High priority)	Critically important (High priority)	Critically important (Highest priority)	Critically important (Highest priority)	Highly important	Highly important	Important	Critically important (High priority)

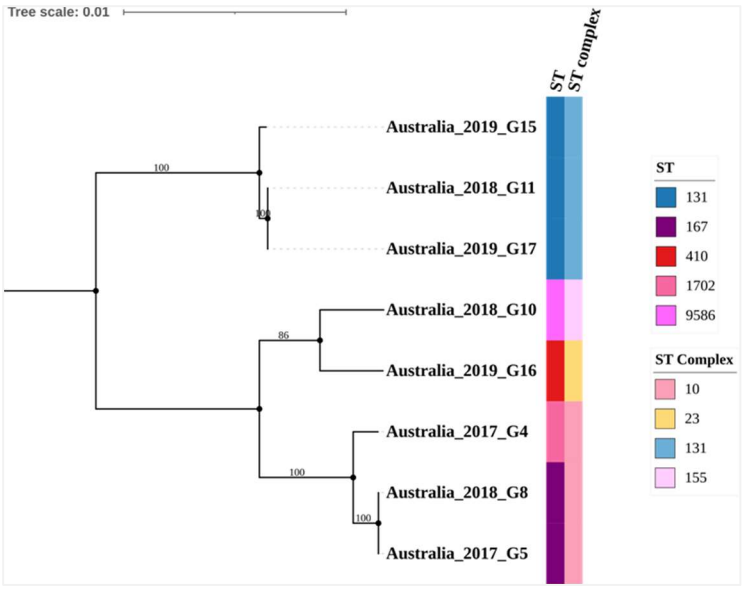
Acronym: R = resistant, **I** = intermediate, and **S** = sensitive.
* MIC (μg/mL).
# Inhibition zone diameter (nearest whole millimetre (mm)).

3.3.2 Genome and phylogenetic analysis

The general genomic characteristics of the *E. coli* isolates from wastewater are given in Table S3.2. Quality-filtered paired-end reads were assembled into between 73 and 469 contigs, with an N50 between 61 and 213 kilobase pairs (kbp). The approximate size of the assembled genomes ranges from 4.87 to 6.13 megabase pairs (Mbp), with GC content between 50.46 and 51.81% (Table S3.2). Annotation of the genomes predicted between 4,562 and 6,002 genes with coding sequence (CDS) between 4,498 and 5,943 (Table S3.2).

Phylogenetic analysis of wastewater isolates based on Achtman's MLST scheme (Wirth et al., 2006) revealed diverse STs (Figure 3.1a). Further resolution of the tree using 3,289 concatenated core CDS demonstrated concordance with the MLST-based clustering (Figure 3.1b). Isolates G11, G15, and G17 were closely related and thus likely belong to the globally disseminated, highly virulent ST131 clones (Peirano et al., 2010; Price et al., 2013; Mathers et al., 2015) and their clonal complexes (Figure 3.1). Isolate G10 belonged to ST9586 within the ST155 clonal complex and was unique to Australia, as further discussed in the subsequent sections (Figure 3.1, Figure 3.4). The G16 isolate belonged to the internationally emerging high-risk ST410 clones of the ST23 clonal complexes (Schaufler et al., 2016; Roer et al., 2018; Feng et al., 2019) (Figure 3.1). The G5 and G8 isolates belonged to the ST167 cluster and were closely related to the ST1702 G4 isolate (Figure 3.1). G4, G5, and G8 belonged to the ST10 clonal complex (Figure 3.1). ST167 was found to have emerged from ST10 clones and has frequently been reported worldwide and is commonly associated with carbapenem resistance in humans and other animals (Abraham et al., 2015; Zong et al., 2018; Garcia-Fernandez et al., 2020). These lineages are further discussed in the following sections.

(a)



(b)

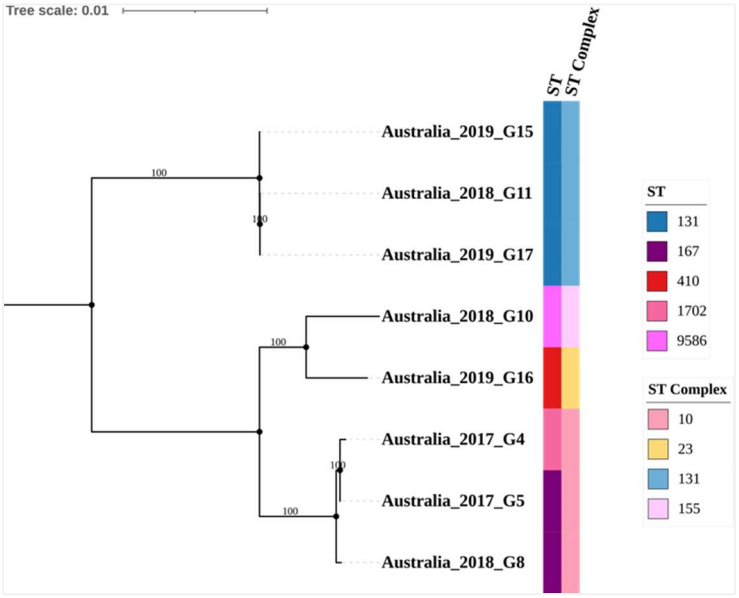


Figure 3.1 Maximum likelihood phylogenetic trees of *E. coli* isolates from wastewater (n = 8). (a) The tree was inferred from the alignment of concatenated MLST gene alleles (*adk–fumC–gyrB–icd–mdh–purA–recA*) extracted from the genome assembly applying the GTR-Gamma model with 1,000 bootstraps in RAxML and is midpoint rooted. (b) The phylogeny was inferred from an alignment of concatenated core CDS (n = 3,289) determined by Roary and RAxML with the GTR-Gamma model and 1,000 bootstrap iterations, and is midpoint rooted. The tree nodes are labelled with the origin, year of isolation, and ID of the isolates. The coloured strips indicate different sequence types (ST) and the respective ST complexes of the isolates.

3.3.2.1 Wastewater isolates are related to pandemic ST167 and emerging ST1702 strains from human and non-human sources

The Roary analysis identified around 3,417 core CDS (Supplementary Figure S3.1) in XDR CR-EC isolates G4, G5, and G8 from wastewater and randomly selected global isolates (n = 306) belonging to ST167 and ST1702 of the ST10 clonal complex. The phylogenetic tree based on the core CDS demonstrated multiple clades of ST167 isolated from human and non-human sources from around the globe over the past 20 years or so (United_States_1999-SRR7042020), and since 2007 also from Australia (Australia_2007-SRR6455985 and Australia_2007-SRR11495756) (Figure S3.2). ST1702 isolates are rarely found and, if so, mostly from humans. The isolates seemed to have emerged in recent years (2015) in some countries, including Australia (2016), from the widespread ST167 clades (Figure S3.2, Figure 3.2). A high prevalence of resistance genes against β-lactams, including carbapenem (*bla*$_{\text{NDM-5}}$, *bla*$_{\text{OXA-1}}$, and *bla*$_{\text{CTX-M-15}}$), aminoglycosides (*rmtB*, *aadA5*, *aadA2*, *aac(6')-lb-cr, aph(3')-Ib,* and *aph(6)-Id*), fluoroquinolone (*aac(6')-lb-cr* and *qnrS1*), sulfonamides (*sul1*), and trimethoprim (*dfrA12*) were observed in the ST167 and ST1702 clusters (Figure S3.2, Figure 3.2).

It was also apparent that the XDR wastewater isolate G5 (Australia_2017_G5) from the ST167 cluster was closely related to MDR-type, carbapenemase-producing isolates from Finnish dogs in 2015 (Sequence Read Archive accession numbers, SRR6451281, SRR6451282, and SRR6451284 to SRR6451286) and from Chinese humans in 2017 (SRR11514723 and SRR11514626) (Figure 3.2). In addition, isolate G5 was found to be related to an Australian human isolate from 2016 (SRR9734793) (Figure 3.2). Similarly, the XDR isolate G8 (Australia_2018_G8) from the ST167 cluster was closely related to human isolates from overseas, such as an MDR-type, carbapenemase-producing isolate from Bangladesh found in 2015 (ERR5685099) and a non-MDR isolate from the United States in 2017 (SRR9988394) (Figure 3.2). However, distinct patterns of resistance gene carriage against multiple critically important antibiotic classes were observed for XDR G5 and G8 isolates compared to related isolates (Figure 3.2).

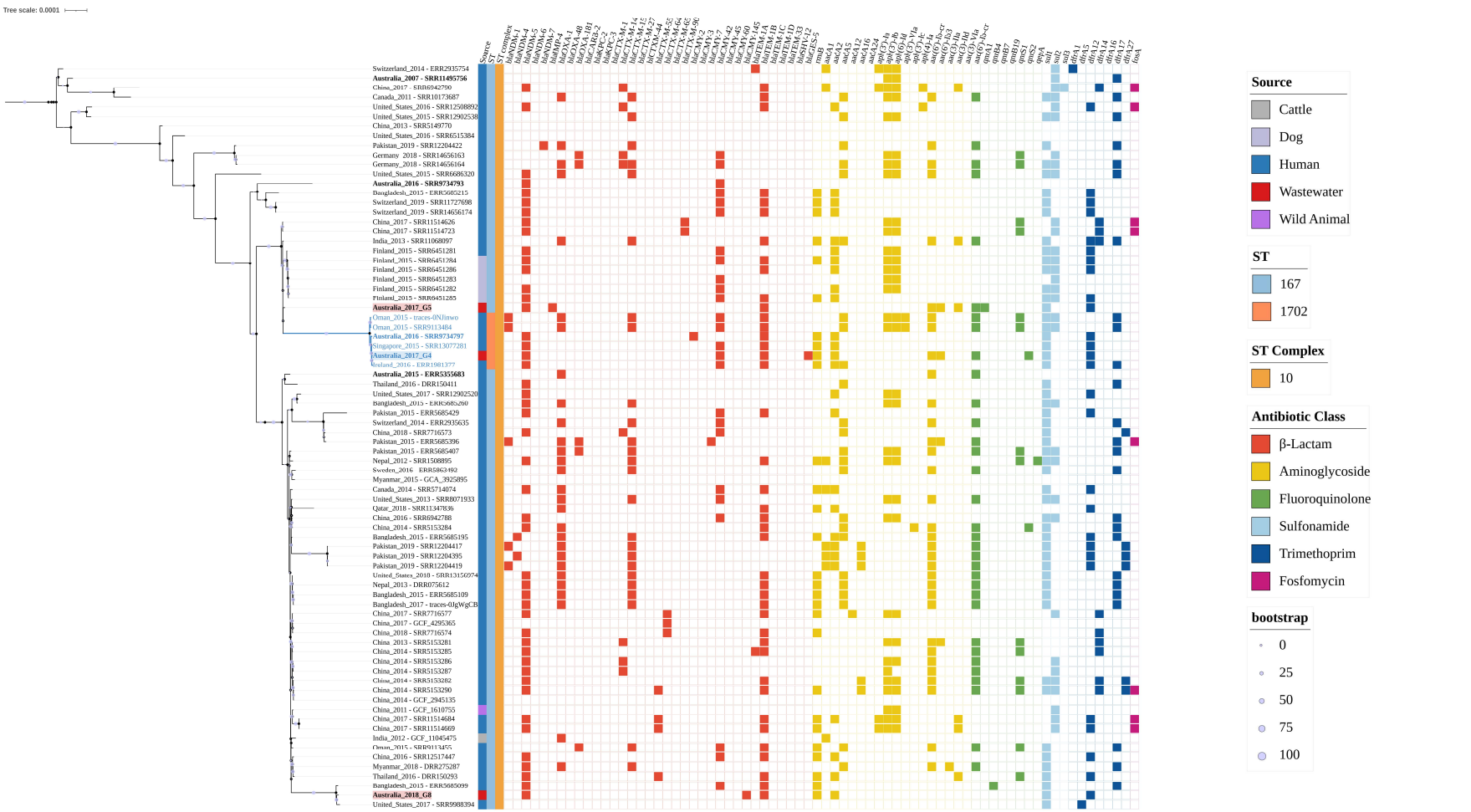


Figure 3.2 Maximum likelihood phylogeny of the ST10 clonal complex (for a larger phylogenetic tree of 309 ST10, ST167, and ST1702 *E. coli* isolates, see Figure S3.2). The phylogeny was inferred from the alignment of the concatenated core CDS (n = 3,417) determined by Roary and RAxML using the GTR-Gamma model and 100 bootstrap iterations. The tree nodes are labelled with the origin, year of isolation, and accession number of the isolates. The wastewater isolates are in bold and highlighted in pink for ST167 and blue for ST1702. The remaining Australian isolates from Enterobase are in bold. The source of the isolates, sequence types (ST), ST complexes, and acquired resistance genes against clinically important antibiotic classes are annotated on the coloured strips according to the given key.

MDR and XDR ESBL-producing ST167 has been reported as the most frequently isolated *E. coli* clone over the past seven years in human clinical samples, as well as in wild and domestic animals, including livestock and dairy from Chile (Báez et al., 2015), Germany (Fischer et al., 2014; Irrgang et al., 2017), Taiwan (Su et al., 2016; Lee et al., 2017), and Tunisia (Dziri et al., 2016; Mani et al., 2017). In addition, carbapenemase-producing ST167 *E. coli* clones were reported to be the second most prevalent clones, ST131 being the most prevalent, from human clinical isolates in India (Devanga Ragupathi et al., 2017), China (Chen et al., 2016; Huang et al., 2016; Shen et al., 2016; Zhu et al., 2016; Zhang et al., 2017), France (Cuzon et al., 2015), Italy (Errico et al., 2018), Spain (Diestra et al., 2009; Sánchez-Benito et al., 2017), Iran (Solgi et al., 2017), Tunisia (Dziri et al., 2016), and Romania (Usein et al., 2016). Of particular concern is the recent report on the possible transmission of carbapenemase ($bla_{\text{NDM-5}}$) producing ST167 clones between dogs and humans within a familiar context in Finland (Grönthal et al., 2018) and in Italy (Alba et al., 2021). With the potential of being a dominant clone and possible interspecies transmission, the presence of the carbapenemase-producing XDR *E. coli* ST167 clone in Australia (Figure S3.2, Figure 3.2) is concerning. Therefore, surveillance targeting antibiotic resistance in both human and non-human sources, such as those based on wastewater, is important to monitor and control the emergence and transmission of this high-risk clone.

The XDR ST1702 isolate G4 (Australia_2017_G4) within the ST167 clonal cluster was closely related mainly to recently emerged MDR-type, carbapenemase-producing human isolates from Singapore found in 2015 (SRR13077281) and Ireland in 2016 (ERR1981377) but also to a human isolate from Australia in 2016 (SRR9734797) (Figure 3.2). The ST1702 cluster is characterised by the high prevalence of β-lactams, including carbapenem ($bla_{\text{NDM-5}}$, $bla_{\text{CMY-2}}$, and $bla_{\text{TEM-1B}}$), aminoglycosides (*rmtB* and *aadA2*), sulfonamides (*sul1*), and trimethoprim (*dfrA12*) resistance genes (Figure 3.2). Detection of emerging ST1702 *E. coli* from wastewater with multiple acquired antibiotic resistance genes again highlights the potential of wastewater testing in monitoring emerging XDR clones circulating in the community.

3.3.2.2 Wastewater isolate is associated with pandemic ST410 strains from human sources

A total of 3,467 core CDS (Figure S3.3) were identified by Roary analysis in the XDR CR-EC isolate G16 from wastewater and randomly selected global isolates (n = 207) belonging to ST410 and closely related members of the ST23 clonal complex. Phylogenetic analysis based on the core CDS demonstrated multiple clades of ST410 and a separate cluster of ST23 isolates (Figure S3.4). The clustering of isolates was irrespective of the isolates' source, collection year, and geographic location (Figure S3.4).

From the phylogenetic analysis (Figure S3.4), it was evident that the carbapenemase-producing ST410 isolate from humans has been in Australia since 2008 (SRR6455995). The XDR G16 isolate from wastewater in 2019 (Australia_2019_G16) was closely related to other MDR-type, carbapenemase-producing isolates from Australian humans in 2013 (SRR9734586), 2014 (SRR9734649), 2015 (SRR9734564), 2016 (SRR9734632), and 2017 (SRR10126894) (Figure 3.3). The cluster containing G16 (Figure 3.3) and other globally disseminated human and non-human isolates was characterised by the high prevalence of multiple resistance genes against critically important antibiotics such as β-lactams, including carbapenem (*bla*$_{\text{OXA-181}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{CMY-2}}$, *bla*$_{\text{TEM-1B}}$), aminoglycosides (*aadA5*, *aph(3')-Ib, aph(6)-Id, aac(6')-lb-cr,* and *aac(3)-IId*), fluoroquinolones (*aac(6')-lb-cr*), sulfonamides (*sul1*), and trimethoprim (*dfrA17*).

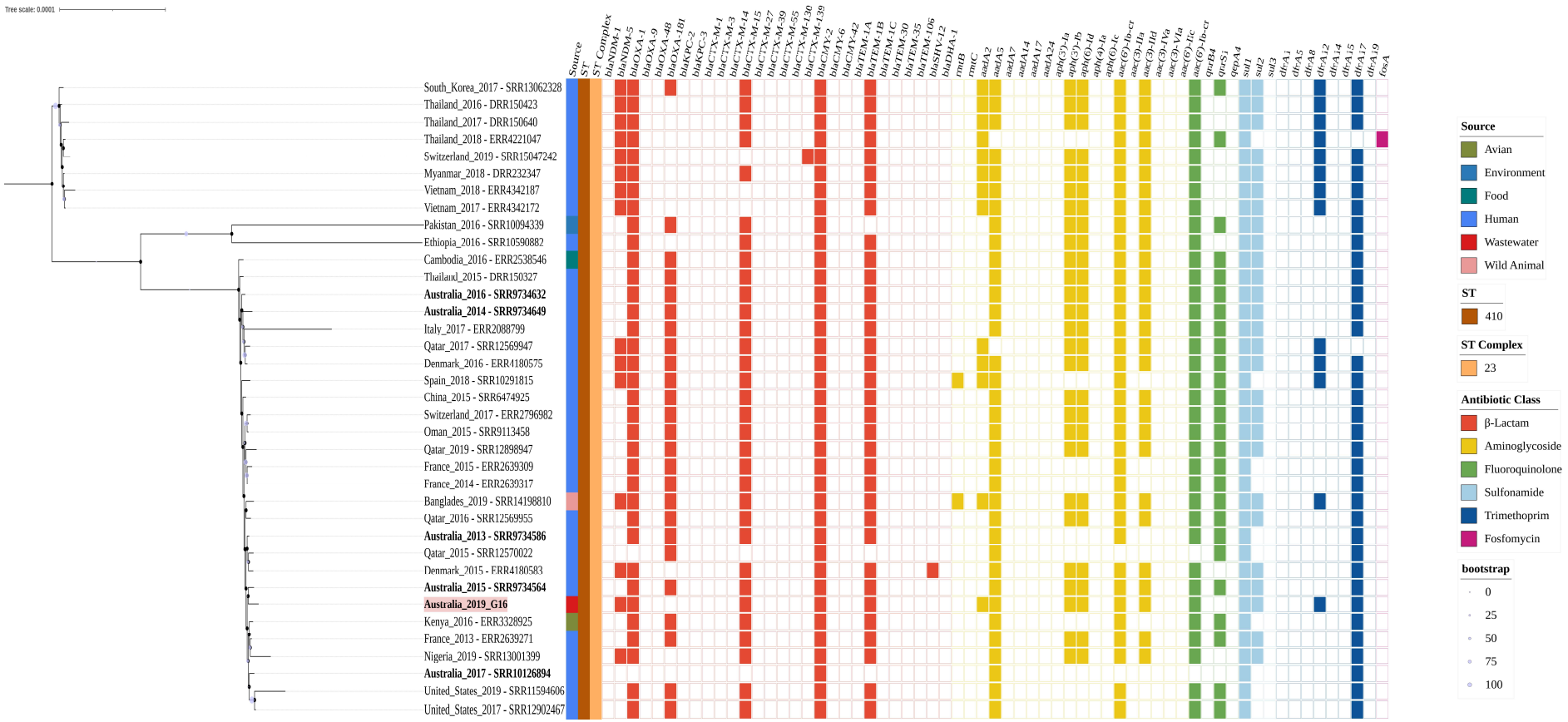


Figure 3.3 Maximum likelihood phylogeny of the ST23 clonal complex (for a larger phylogenetic tree of 208 ST410 and ST23 *E. coli* isolates, see Figure S3.4). The phylogeny was inferred from the alignment of the concatenated core CDS (n = 3,467) determined by Roary and RAxML using the GTR-Gamma model and 100 bootstrap iterations. The tree nodes are labelled with the origin, year of isolation, and accession number of the isolates. The wastewater isolate is in bold and highlighted in pink, and the remaining Australian isolates from Enterobase are in bold. The source of the isolates, sequence types (ST), ST complexes, and acquired resistance genes against different clinically important antibiotic classes are annotated on the coloured strips according to the given key.

ST410 has been described as an international high-risk clone owing to its potential for global dissemination, persistence in human and non-human hosts, ease of transmission between hosts, and acquisition of resistance determinants against various antibiotic classes, including carbapenem (Schaufler et al., 2016; Roer et al., 2018; Feng et al., 2019). The ST410 clone has also been found to persist and cause recurrent infections in humans, including bloodstream infections with the ability of patient-to-patient transmission in healthcare settings (Roer et al., 2018). In addition, clonal dissemination of *bla*$_{\text{CTX-M-15}}$ -type β-lactamase producing ST410 clones was observed between humans, livestock, and companion animals in Germany (Falgenhauer et al., 2016). Frequent isolation of the MDR and carbapenemase-producing ST410 clone from human sources in Australia (Figure 3.3) requires close monitoring to control the spread of this high-risk clone in the community. Future surveillance should also target non-human sources for this widely disseminated clone to identify possible transmission sources to support effective control and prevention strategies.

3.3.2.3 First detection of a likely non-human ST9586 isolate in Australia

Around 3,702 core CDS (Figure S3.5) were identified by Roary analysis in the MDR ESBL-EC isolate G10 from wastewater, other global isolates belonging to the ST9586 clone (n = 2) and randomly selected closely related ST678 isolates (n = 19). Phylogenetic analysis using the core CDS demonstrated a distinct clustering of ST9586 isolates mostly from the non-human origin (Figure 3.4). Furthermore, annotation of the phylogeny with antibiotic resistance genes indicates an independent acquisition of the *bla*$_{\text{CTX-M-55}}$ -type β-lactamase and plasmid-mediated quinolone resistance gene *qnrS1* by MDR isolates in the ST9586 clusters (Figure 3.4). To the best of my knowledge, ST9586 isolates have never been reported in Australia. Moreover, it was evident from the phylogeny that the ST9586 isolate G10 (Australia_2018_G10) was very similar to recently isolated environmental and poultry strains from Japan (DRR199744) and the United States (SRR9201955), respectively (Figure 3.4). This finding highlights the potential of routine wastewater-based monitoring for the early detection of emerging MDR clones before they might appear in clinical settings.

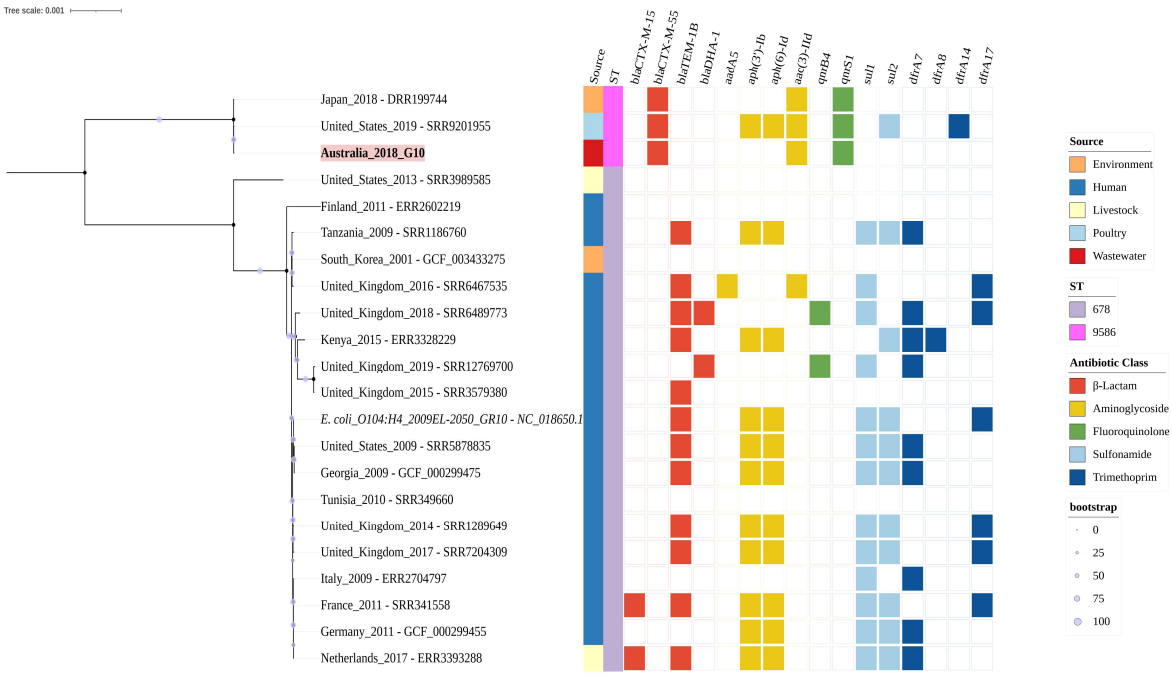


Figure 3.4 The maximum likelihood phylogeny of ST9586 and closely related ST678 *E. coli* isolates (n = 22) was inferred from the alignment of the concatenated core CDS (n = 3,702) determined by Roary and RAxML using the GTR-Gamma model and 1,000 bootstrap iterations. The tree is midpoint rooted, and the tree nodes are labelled with the origin, year of isolation, and accession number of the isolates. The wastewater isolate is in bold and highlighted in pink. The source of the isolates, sequence types (ST) and acquired resistance genes against clinically important antibiotic classes are annotated on the coloured strips according to the given key.

3.3.2.4 Wastewater isolates are related to pandemic ST131 strains from human sources

Roary analysis identified around 3,297 core CDS (Figure S3.6) in the MDR ESBL-EC isolates G11, G15, and G17 from wastewater and randomly selected global isolates (n = 502) belonged to the ST131 clone. Phylogenetic analysis based on the core CDS indicated that multiple ST131 clades are widely distributed between human and non-human sources with no distinct geographical and temporal clustering (Figure S3.7). However, the presence of resistance genes against clinically significant antibiotic classes showed a distinct clustering of the carbapenemase-producing ST131 isolates (Figure S3.7). Furthermore, it was evident from the phylogeny that the ST131 isolates have been around the world since 1982 (United_States_1982-SRR6986385) and in Australia since 2005 (Australia_2005-SRR11608164 and Australia_2005-ERR537636).

The wastewater isolates G11 (Australia_2018_G11), G15 (Australia_2019_G15), and G17 (Australia_2019_G17) were found to be grouped in the phylogeny with other MDR and mostly non-carbapenemase producers from human and non-human sources from Australia and around the world (Figure S3.7, Figure 3.5). It was also evident that these wastewater isolates were closely clustered in the phylogeny with an MDR Australian isolate from a patient with bloodstream infection in 2015 (SRR5936479) carrying resistance genes against β-lactams (*bla*$_{\text{CTX-M-27}}$ and *bla*$_{\text{TEM-1B}}$), aminoglycosides (*aadA5, aph(3')-Ib, aph(6)-Id,* and *aac(3)-IId*), sulfonamides (*sul1* and *sul2*), and trimethoprim (*dfrA17*) (Figure 3.5). The G15 isolate found in 2019 was found to have resistance genes identical to those carried by the closely related Australian isolate found in 2015 (SRR5936479) (Figure 3.5). The G17 isolate found in 2019 possesses more resistance genes against β-lactams (*bla*$_{\text{CMY-2}}$, *bla*$_{\text{CMY-58}}$, *bla*$_{\text{CTX-M-27}}$, and *bla*$_{\text{TEM-1C}}$), aminoglycosides (*aadA5, aph(3')-Ib, aph(6)-Id,* and *aac(3)-IId*), sulfonamides (*sul* and *sul2*), and trimethoprim (*dfrA17*) compared to closely clustered human isolates from Australia found in 2015 (SRR5936479) (Figure 3.5). Whereas isolate G11 in 2018 carried fewer resistance genes (only against β-lactams, i.e. *bla*$_{\text{CMY-2}}$, *bla*$_{\text{CTX-M-27}}$, and *bla*$_{\text{TEM-1C}}$) than the closely related Australian strain isolated in 2015 (SRR5936479) (Figure 3.5).

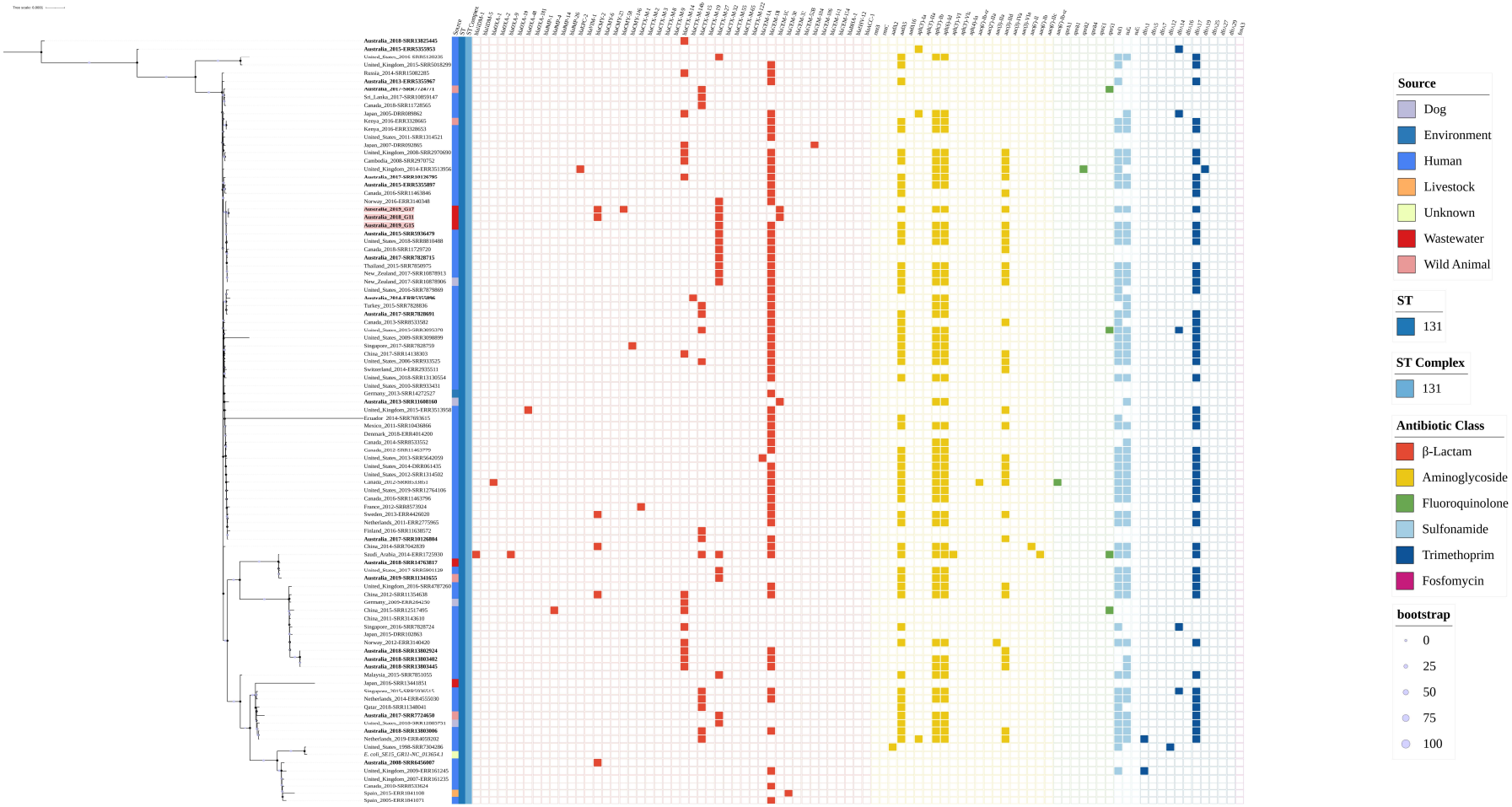


Figure 3.5 Maximum likelihood phylogeny of the ST131 clonal complex (for a larger phylogeny of 505 ST131 *E. coli* isolates, see Figure S3.7). The phylogeny was inferred from the alignment of the concatenated core CDS (n = 3,297) determined by Roary and RAxML using the GTR-Gamma model and 100 bootstrap iterations. The tree nodes are labelled with the origin, year of isolation, and accession number of the isolates. The wastewater isolates are in bold and highlighted in pink, and the remaining Australian isolates from Enterobase are in bold. The source of the isolates, sequence types (ST), ST complexes, and acquired resistance genes against different clinically important antibiotic classes are annotated on the coloured strips according to the given key.

The ST131 clone is the predominant *E. coli* clone disseminated worldwide, characterised by the high virulence potential and is frequently associated with bacteraemia and healthcare- and community-acquired urinary tract infections (Nicolas-Chanoine et al., 2014; Petty et al., 2014; Decano & Downing, 2019; Pitout et al., 2020). ST131 clones typically carry β-lactamase, such as CTX-M-15, and fluoroquinolone resistance genes (Banerjee et al., 2014; ECDC, 2017; Findlay et al., 2020) and less frequently carbapenemase genes (Peirano et al., 2011; Peirano et al., 2014). Although humans were identified as the primary reservoir, ST131 clones have also been less frequently identified from a range of non-human sources, including animals such as wild, companion, livestock, poultry, avian, primate, food production; soil; wastewater and beach water (Vignaroli et al., 2013; Said et al., 2015; McNally et al., 2016; Jamborova et al., 2018; Finn et al., 2020; Pitout et al., 2020). In addition, the transmission of the MDR ST131 clone between members within the same household (Ender et al., 2009; Johnson et al., 2010; Owens et al., 2011; Madigan et al., 2015) and between family members and companion animals (Johnosn et al., 2009; Johnson et al., 2016) has been documented. Several studies in Australia also reported MDR ST131 as a dominant clone of human bloodstream and urinary tract infections (Kudinha et al., 2013; Rogers et al., 2015; Harris et al., 2017; Hastak et al., 2020), wild animals (Mukerji et al., 2019) and companion animal (Platell et al., 2010; Guo et al., 2013), with interspecies transmission (Kidsley et al., 2020). Therefore, continuous monitoring of the highly virulent MDR ST131 clone in humans and a diverse range of non-human sources is required to track and control the emergence and spread of this clone.

Overall, the phylogenetic analysis showed that wastewater picked up strains mostly from human sources, but also strains that have previously been predominantly found in non-human sources. In addition, I found that wastewater testing has the ability to detect new strains that have not previously been found in the local Australian setting.

3.3.3 Genetic determinants of antibiotic resistance

As the previous section has highlighted that the wastewater isolates possess a number of unique antibiotic resistance profiles, I next examined the molecular mechanisms underpinning resistance. ResFinder predicted multiple antibiotic resistance genes against clinically significant antibiotic classes from the draft genome of the XDR CR-EC and

MDR ESBL-EC isolates from wastewater (Table 3.2). The detailed resistance mechanisms for each antibiotic class are discussed in the following sections.

3.3.3.1 Resistance to β-lactams is mainly mediated by diverse genes encoding ESBLs and carbapenemases

Carbapenem (i.e. meropenem) resistance in the XDR CR-EC isolates G4, G8, and G16 was mainly mediated by the carriage of NDM-5-type carbapenemase (Table 3.2) and the co-occurrence of NDM-5 and IMP-4 in the isolate G5 (Table 3.2). These carbapenemases encoding genes are consistent with the resistance of the CR-EC isolates to all other tested β-lactam antibiotics such as aminopenicillin (i.e. ampicillin), first-, second-, third-, and fourth-generation cephalosporins (i.e. cefazolin, ceftriaxone, ceftazidime, and cefepime), cephamycin (i.e. cefoxitin), and penicillins and β-lactam inhibitors (i.e. amoxicillin/clavulanic acid, piperacillin/tazobactam, and ticarcillin/clavulanic acid) (Table 3.2). In addition to carbapenemase, these isolates carried TEM-1B-, GES-5- and OXA-1-type β-lactamases, CTX-M-15-type ESBL, and CMY-type AmpC β-lactamases in variable combinations (Table 3.2).

Table 3.2 Genetic characteristics of the carbapenem-resistant *E. coli* (CR-EC) and extended-spectrum β-lactamases-producing *E. coli* (ESBL-EC) isolates.

Isolates	MLST types	Phenotypes	Antibiotic resistance pattern[#]	Antibiotic resistance genes	Virulence-associated genes	Plasmid replicons
G4	1702	CR-EC	AMP, FOX, CFZ, CAZ, CRO, FEP, MEM, AMC, TZP, TIM, GEN, TOB, AMK, CIP, NOR TMP, SXT	*bla*$_{NDM-5}$, *bla*$_{CMY-42}$, *bla*$_{GES-5}$, *bla*$_{TEM-1B}$, *rmtB*, *aac(6')-Ib3*, *aac(6')-Ib-cr*, *qnrS2*, *gyrA* (S83L, D87N), *parC* (S80I, P578L), *parE* (S458A), *dfrA12, sul1*	*capU*, *fimH*, *fyuA*, *hra*, *irp2*, *iss*, *terC*, *traT*	Col (pHAD28), ColRNAI, IncFIA, IncFII, IncI (Gamma), IncQ2
G5	167	CR-EC	AMP, FOX, CFZ, CAZ, CRO, FEP, MEM, AMC, TZP, TIM, GEN, TOB, AMK, CIP, NOR, TMP, SXT	*bla*$_{NDM-5}$, *bla*$_{IMP-4}$, *bla*$_{TEM-1B}$, *aac(6')-Ib3*, *aac(3)-IId*, *aac(6')-Ib-cr*, *qnrA1, gyrA* (S83L, D87N), *parC* (S80I, P578L), *parE* (S458A), *dfrA12, sul1*	*capU*, *fimH*, *fyuA*, *hra*, *irp2*, *iss*, *terC*, *traT*	Col156, Col (pHAD28), Col440I, Col440II, ColRNAI, FII (pBK30683), IncFIA, IncFIB (AP001918), IncFIB (K), IncFII, IncFII (K), IncM2, IncR
G8	167	CR-EC	AMP, FOX, CFZ, CAZ, CRO, FEP, MEM, AMC, TZP, TIM, GEN, TOB, AMK, CIP, NOR, TMP, SXT, NIT	*bla*$_{NDM-5}$, *bla*$_{CMY-145}$, *bla*$_{TEM-1B}$, *rmtB, gyrA* (S83L, D87N), *parC* (S80I, P578L), *parE* (S458A), *dfrA12*, *sul1*, *nfsA*, *nfsB*	*capU*, *gad*, *hra*, *iss*, *iucC, iutA*, *terC*, *traT*	IncF1A, IncFIB (AP001918), IncFII, IncI (Gamma)
G16	410	CR-EC	AMP, FOX, CFZ, CAZ, CRO, FEP, MEM, AMC, TZP, TIM, GEN, TOB, CIP, NOR, TMP, SXT	*bla*$_{NDM-5}$, *bla*$_{OXA-1}$, *bla*$_{CMY-2}$, *bla*$_{TEM-1B}$, *bla*$_{CTX-M-15}$, *aac(3)-IId, aac(6')-Ib-cr*, *gyrA* (S83L, D87N), *parC* (S80I), *parE* (S458A), *dfrA12*, *dfrA17, sul1*, *sul2*	*astA*, *fimH*, *gad*, *hra*, *lpfA*, *terC*	Col (BS512), IncFIA, IncFIB (AP001918), IncFII (pAMA1167-NDM-5), IncQ1

G10	9586	ESBL-EC	AMP, FOX, CFZ, CAZ, CRO, FEP, AMC, GEN, NIT	*bla*$_{CTX-M-55}$, *ampC*-promoter (-42, C->T), *aac(3)-IId*, *qnrS1*, *nfsA*, *nfsB*	*etsC*, *fyuA*, *gad*, *hlyF*, *irp2*, *iss*, *iucC*, *iutA*, *lpfA*, *ompT*, *sitA*, *terC*, *traT*	Col440II, Col (pHAD28), ColpVC, IncFIA, IncFIB (AP001918), IncFII
G11	131	ESBL-EC	AMP, FOX, CFZ, CAZ, CRO, FEP, AMC, TZP, TIM, CIP, NIT	*bla*$_{CMY-2}$, *bla*$_{TEM-1C}$, *bla*$_{CTX-M-27}$, *gyrA* (S83L, A828S), *gyrB* (A618T), *parC* (A117E, D475E, T718A), *parE* (V136I, I529L), *nfsA, nfsB*	*chuA*, *fimH*, *fyuA*, *gad*, *iha*, *irp2*, *iucC*, *iutA*, *kpsE*, *kpsMII_K5*, *ompT*, *papA_F43*, *sat*, *senB*, *terC*, *traT*, *usp*, *yfcV*	Col156, IncFIB (AP001918), IncFII (29), IncFII (pRSB107), IncI (Gamma)
G15	131	ESBL-EC	AMP, FOX, CFZ, CAZ, CRO, FEP, GEN, TMP, SXT, NIT	*bla*$_{TEM-1B}$, *bla*$_{CTX-M-27}$, *aac(3)-IId*, *gyrA* (S83L, A828S), *gyrB* (A618T), *parC* (D475E, T718A), *parE* (V136I, I529L), *dfrA17, sul1, sul2, nfsA, nfsB*	*chuA*, *cnf1*, *fimH*, *fyuA*, *gad*, *iha*, *irp2*, *iucC*, *iutA*, *kpsE*, *kpsMII_K5*, *ompT*, *papA_F43*, *papC*, *sat*, *senB*, *sitA*, *terC*, *traT*, *usp*, *yfcV*	Col156, IncFIB (AP001918), IncFII (29)
G17	131	ESBL-EC	AMP, FOX, CFZ, CAZ, CRO, FEP, AMC, TIM, GEN, CIP, TMP, SXT, NIT	*bla*$_{CMY-2}$, *bla*$_{CMY-58}$, *bla*$_{TEM-1C}$, *bla*$_{CTX-M-27}$, *aac(3)-IId*, *gyrA* (S83L, A828S), *gyrB* (A618T), *parC* (A117E, D475E, T718A), *parE* (V136I, I529L), *dfrA17, sul1, sul2, nfsA, nfsB*	*chuA*, *fimH*, *fyuA*, *gad*, *iha*, *irp2*, *kpsE*, *kpsMII_K5*, *ompT*, *papA_F43*, *senB*, *terC*, *traT*, *usp*, *yfcV*	Col156, IncFIB (AP001918), IncFII (29), IncFII (pRSB107), IncI (Gamma)

[#] Here, **AMP** = Ampicillin, **FOX** = Cefoxitin, **CFZ** = Cefazolin, **CAZ** = Ceftazidime, **CRO** = Ceftriaxone, **FEP** = Cefepime, **MEM** = Meropenem, **AMC** = Amoxicillin/clavulanic acid, **TZP** = Piperacillin/tazobactam, **TIM** = Ticarcillin/clavulanic acid, **GEN** = Gentamicin, **TOB** =Tobramycin, **AMK** = Amikacin, **CIP** = Ciprofloxacin, **NOR** = Norfloxacin, **TMP** = Trimethoprim, **SXT** = Trimethoprim-Sulfamethoxazole, **NIT** = Nitrofurantoin.

Furthermore, several deleterious mutations were observed in the *ompC* gene of CR-EC isolates (Table S3.3) that could confer carbapenem resistance when combined with ESBL and/or high level of AmpC production, as previously reported (Stapleton et al., 1999; Martínez-Martínez et al., 2000; Dé et al., 2001; Liu et al., 2012). Among the 35 non-synonymous single nucleotide polymorphisms (SNPs) in the OmpC of isolates G4, G5, and G8, only N165D in one of the transmembrane β-strand (from 164 to 171 amino acid (aa) residues); G190R and R191D in the β-strands connecting the extracellular domain (172-200 aa); D208N in the β-strands connecting the periplasmic domain (208-211 aa); and D225W in the β-strands connecting the extracellular domain (220-241 aa) were predicted to be deleterious by PROVEAN analysis. In the OmpC of isolate G16, two of the ten non-synonymous SNPs (D192G and R195L) in the β-strands connecting the extracellular domain (172-200 aa) were predicted to have a deleterious effect. No deleterious mutations were found in another major porin, OmpF (Nikaido et al., 1994; Pagès et al., 2008; Delcour et al., 2009), and the two-component system EnvZ/OmpR that regulates OmpC and OmpF porin expression (Mizuno & Mizushima, 1987; Slauch et al., 1988) in CR-EC isolates that could still allow the transport of carbapenem in the cell. However, the presence of different carbapenemases could hydrolyse carbapenem inside the cell. Therefore, the carbapenem resistance mechanisms may be attributed mainly to the production of different carbapenemases in CR-EC isolates. Deleterious mutations in OmpC and the production of AmpC and ESBL in these isolates might confer additional non-specific resistance to carbapenem.

Resistance to β-lactam antibiotics in ESBL-EC isolates G10, G11, G15, and G17 was mediated mainly by various ESBL and AmpC β-lactamase enzymes (Table 3.2). In these isolates, resistance to ampicillin and cephalosporins was explained by the presence of CTX-M-type ESBL (Table 3.2). In addition, isolates G11, G15, and G17 carried a TEM-type β-lactamase (Table 3.2). In G11 and G17 isolates, resistance to cefoxitin, amoxicillin/clavulanic acid, piperacillin/tazobactam, and ticarcillin/clavulanic acid was attributed to the carriage of CMY-2-type AmpC β-lactamases (Table 3.2). In isolate G10, potential upregulating mutations in the promoter region of the *ampC* gene (-42, C->T) might explain resistance to cefoxitin (Caroff et al., 1999; Mulvey et al., 2005; Peter-Getzlaff et al., 2011) and amoxicillin/clavulanic acid (Table 3.2). Although isolate G15 was phenotypically detected as cefoxitin resistant, no previously identified mutations that

upregulated the *ampC* gene (Caroff et al., 1999; Nelson & Elisha 1999; Mulvey et al., 2005; Peter-Getzlaff et al., 2011) and CMY-type AmpC β-lactamases were observed, indicating an unknown mechanism of resistance to cephamycin (i.e. cefoxitin).

Interestingly, several functionally deleterious mutations were also predicted for OmpC of carbapenem-susceptible ESBL-EC isolates (Table S3.3). In isolate G10, an insertion of 18 bp between nucleotides 690-691 that added six amino acids (Ins GLNGYG (229-230)) to the extracellular domain (220-241 aa), and one of the 21 non-synonymous SNPs (D192G) in another extracellular domain (172-200 aa) of OmpC were found to be deleterious. In isolates G11, G15 and G17, an insertion of 18 bp between 689-690 positions that added six residues (Ins GLNGYG (229-230)) to the extracellular domain (220-241 aa), and one of the 17 non-synonymous SNPs (D192G) in another extracellular domain (172-200 aa) were predicted to be deleterious. Mutations in OmpF and EnvZ/OmpR were predicted to be neutral in these isolates, and none of the isolates had genes encoding carbapenemase. Therefore, deleterious mutations only in OmpC combined with ESBL production and AmpC hyperproduction in isolate G10; deleterious OmpC mutations along with ESBL in isolate G15; and deleterious mutations in OmpC combined with the production of ESBL and plasmid-mediated AmpC β-lactamases (i.e. CMY-type) in isolates G11 and G17 might not be sufficient to confer a clinically relevant level of carbapenem resistance (Table 3.2, Table S3.3).

3.3.3.2 Aminoglycoside resistance is conferred mainly by genes encoding various aminoglycoside-modifying enzymes

The most prevalent and clinically relevant mechanism of aminoglycoside resistance in *E. coli* is the enzymatic inactivation of the antibiotic by acetylation (aminoglycoside acetyltransferases, AAC), adenylation (aminoglycoside nucleotidyltransferases, ANT) and phosphorylation (aminoglycoside phosphotransferases, APH) (Shaw et al., 1993; Ramirez & Tolmasky, 2010; Ramirez et al., 2013; Wachino et al., 2020). Another globally disseminated and clinically significant mechanism of aminoglycoside resistance is the acquisition of plasmid-mediated 16S ribosomal RNA (rRNA) methyltransferases (16S RMTases) such as *armA, rmtB-H,* and *npmA* (Galimand et al., 2003; Doi & Arakawa, 2007; Doi et al., 2016; Wachino et al., 2020). The 16S RMTases can confer high-level resistance to almost all aminoglycosides by methylating a nucleotide in the

aminoglycoside binding region located in the aminoacyl-transfer RNA recognition site (i.e. A-site) of 16S rRNA using the S-adenosyl-L-methionine as a cofactor (Galimand et al., 2003; Doi et al., 2016; Wachino et al., 2020).

Resistance to clinically significant aminoglycosides such as gentamicin, tobramycin, and amikacin in this study's isolates was mediated by various aminoglycoside acetyltransferases and 16S RMTase encoding genes (Table 3.2). Amikacin resistance in CR-EC isolates G4 and G8 was explained by the carriage of a 16S RMTases encoding gene (i.e. *rmtB*) (Table 3.2), which also would render these isolates resistant to tobramycin and gentamicin, as previously documented (Galimand et al., 2003; Doi & Arakawa, 2007; Doi et al., 2016; Wachino et al., 2020). Isolate G4 also carried an *aac(6')-Ib3* gene encoded for the aminoglycoside 6'-N-acetyltransferase type Ib3 and an *aac(6')-Ib-cr* encoding the aminoglycoside 6'-N-acetyltransferase type Ib-cr, capable of conferring resistance to tobramycin and amikacin (Ramirez & Tolmasky, 2010; Kim et al., 2011; Ramirez et al., 2013; Wachino et al., 2020). Likewise, the occurrence of the *aac(6')-Ib3* and *aac(6')-Ib-cr* genes in the CR-EC isolate G5 likely conferred resistance to amikacin and tobramycin, and the carriage of the *aac(3)-IId* gene encoded for the aminoglycoside 3-N-acetyltransferase type IId can explain gentamicin resistance (Table 3.2). In CR-EC isolate G16, the presence of *aac(3)-IId* and *aac(6')-Ib-cr* gene explained the resistance to gentamicin and tobramycin, respectively (Table 3.2). Interestingly, despite the presence of an *aac(6')-Ib-cr* gene, isolate G16 was phenotypically detected as amikacin sensitive (Table 3.1, Table 3.2). The promoter region of the *aac(6')-Ib-cr* gene was found to be unchanged, and no deleterious mutations were found in the gene. Therefore, the mechanism of discordance between amikacin resistance genes and the susceptibility of isolate G16 remains unclear. ESBL-EC isolates G10, G15 and G17 were resistant only to gentamicin, which was correlated with the presence of the *aac(3)-IId* gene (Table 3.2).

3.3.3.3 Fluoroquinolone resistance is mainly attributed to mutations in the genes encoding antibiotic target enzymes

The clinically significant level of fluoroquinolone (i.e. ciprofloxacin, norfloxacin) resistance in *E. coli* is commonly associated with mutations in the antibiotic's target enzymes, such as DNA gyrase encoded by the *gyrA* and *gyrB* genes and topoisomerase

IV encoded by the *parC* and *parE* genes (Ruiz, 2003; Hopkins et al., 2005; Hooper & Jacoby, 2016). Most of these mutations that confer resistance by decreasing the binding of fluoroquinolones to the mutant enzyme-DNA complex are frequently located in a specific region of the DNA gyrase and topoisomerase IV enzymes known as the quinolone resistance-determining region (QRDR) (Yoshida et al., 1990). In *E. coli*, the QRDRs of GyrA (67-106 aa) and ParC (63-102) are located near the N-terminal domain and harbour the ATPase active site, while the QRDRs of GyrB (423-430 and 445-449) and ParE (417-424 and 439-443 aa) are located near the C-terminal DNA-binding domain (Yoshida et al., 1990; Yoshida et al., 1991; Nawaz et al., 2015; Hooper & Jacoby, 2016). In addition, a low-level of fluoroquinolone resistance can also be mediated by plasmid-mediated quinolone resistance (PMQR) determinants, such as *qnrA-D*- and *qnrS*-type genes encoding repetitive peptides that bind to and protect DNA gyrase and topoisomerase IV from quinolone inhibition; the *aac(6')-Ib-cr* gene encoded for an aminoglycoside acetyltransferase that can modify quinolones; and the *qepA*- and *oqxAB*-type genes encoding efflux pumps (Martínez-Martínez et al., 1998; Nordmann & Poirel, 2005; Tran et al., 2005; Robicsek et al., 2006; Hansen et al., 2007; Yamane et al., 2007; Jacoby et al., 2014).

Resistance to ciprofloxacin and norfloxacin in CR-EC isolates G4, G5, G8, and G16 could be mainly attributed to mutations in the QRDR of GyrA and ParC, and outside the QRDR of ParE proteins (Table 3.2). For instance, isolates G4, G5, G8, and G16 have previously reported mutations in GyrA (S83L and D87N), ParC (S80I), and ParE (S458A) that confer resistance (Table 3.2) (Ruiz et al., 2003; Hopkins et al., 2005; Sorlozano et al., 2007; Bansal & Tandon, 2011; Hooper & Jacoby, 2016). A novel deleterious mutation in ParC (P578L) located outside the QRDR region was also found in isolates G4, G5, and G8 (Table 3.2). In addition to chromosomal mutations, isolates G4 and G5 had *aac(6')-Ib-cr* and *qnr*-type PMQR genes, and isolate G8 carried an *aac(6')-Ib-cr* gene (Table 3.2).

Ciprofloxacin resistance in ESBL-EC isolates G11 and G17 was likely mediated by mutations in GyrA (S83L) and ParC (A117E), as previously documented (Table 3.2) (Ruiz et al., 2003; Hopkins et al., 2005; Hooper & Jacoby, 2016). These isolates also had neutral mutations outside the QRDR of GyrA (A828S), GyrB (A618T), ParC (D475E and T718A), and ParE (V136I and I529L), and none had PMQR genes (Table 3.2).

Interestingly, despite carrying these resistant-conferring and neutral mutations, isolates G11 and G17 were phenotypically identified as sensitive to norfloxacin (Table 3.2). The mechanism for this genotype-phenotype discrepancy remains unclear. As isolate G10 had wild-type GyrAB and ParCE, intermediate susceptibility to ciprofloxacin can be explained by the carriage of a *qnrS1* gene, which confers low-level resistance (Martínez-Martínez et al., 2003; Allou et al., 2009; Sato et al., 2013; Jacoby et al., 2014). Although isolate G15 had a resistance mutation in GyrA (S83L), it was sensitive to ciprofloxacin and norfloxacin (Table 3.1, Table 3.2). In addition, this isolate did not have deleterious mutations in the genes encoding GyrB, ParC, and ParE and lacked PMQR genes (Table 3.2). Therefore, the mutation in GyrA alone might not be sufficient to confer a clinically relevant level of fluoroquinolone resistance in isolate G15.

3.3.3.4 Resistance to trimethoprim and sulfamethoxazole is associated with genes encoding antibiotic-resistant variants of the chromosomal target enzymes

In *E. coli*, high-level resistance to sulfonamide (i.e. sulfamethoxazole) is mainly attributed to the acquisition of *sul*-type genes coding for a drug-resistant variant of the chromosomal target enzyme dihydropteroate synthase that catalyses the first step of folate biosynthesis (Huovinen et al., 1995; Sköld, 2001; Bean et al., 2009; Poey et al., 2019). Folate and its derivatives are essential cofactors for the synthesis of nucleotides and amino acids (i.e. methionine, serine, glycine, and histidine) (Green & Matthews, 2007; Naderi & House, 2018). Likewise, a high level of trimethoprim resistance is mediated by *dfr*-type genes coding for a resistant variant of the antibiotic's target enzyme dihydrofolate reductase, which catalyses the final step of folate biosynthesis (Huovinen et al., 1995; Huovinen, 2001; Sköld, 2001). The *sul*- and *dfr*-type genes are often carried on MGEs such as plasmids, integrons, and transposons that contribute to the horizontal spread (Rådström et al., 1991; Recchia & Hall, 1995; Sköld, 2001; White et al., 2001; Bean et al., 2009).

Resistance to trimethoprim in CR-EC isolates G4, G5, G8, and G16 can be explained by the presence of the *dfrA12* gene (Table 3.2), as reported previously (Lee et al, 2001; Sköld, 2001; White et al., 2001; Brolund et al., 2010; Harris et al., 2018). Isolate G4, G5, G8, and G16 also coharboured *dfrA12* and *sul1* genes that might confer resistance to the trimethoprim-sulfamethoxazole combination, as per previous studies (Huovinen, 2001;

Blahna et al., 2006; Ho et al., 2009). The G16 isolate was found to have additional *dfrA17* and *sul2* genes (Table 3.2). Trimethoprim resistance in ESBL-EC isolates G15 and G17 was mediated by the *dfrA17* gene, and trimethoprim-sulfamethoxazole resistance was conferred by the co-occurrence of the *dfrA17* and *sul1, sul2* genes (Table 3.2).

3.3.3.5 Nitrofurantoin resistance is mainly caused by novel mutations in the nitroreductase encoding genes and likely some unknown mechanisms

Nitrofurantoin resistance in *E. coli* is mainly mediated by mutations in the chromosomal genes *nfsA* and *nfsB* that encode for the oxygen-insensitive nitroreductases responsible for reducing nitrofurantoin into toxic intermediate compounds (Sandegren et al., 2008). Nitrofurantoin resistance has also been reported to be caused by the inactivation of the *ribE* gene encoding lumazine synthase, which is responsible for the biogenesis of flavin mononucleotide (FMN), an essential cofactor for NfsA and NfsB (Vervoort et al., 2014). The plasmid-encoded multidrug efflux pump genes *oqxAB*-type have furthermore been reported to be associated with nitrofurantoin resistance (Ho et al., 2015).

As none of the CR-EC and ESBL-EC isolates had *oqxAB*-type genes and mutations in the *ribE* gene, nitrofurantoin resistance is likely caused by mutations in the *nfsA* and *nfsB* genes, as reported previously (Sandegren et al., 2008; Vervoort et al., 2014; Ho et al., 2015; Zhang et al., 2018). Comparison with the wild-type *E. coli* J53 strain (GenBank accession number, AICK00000000.1) revealed several unique or previously reported mutations in the *nfsA* and *nfsB* genes in the eight isolates. For instance, a unique nine-nucleotide (227-235) deletion, resulting in the loss of three amino acids (FWV) at positions 76 to 78, was observed in the *nfsA* gene of the nitrofurantoin-intermediate G4, G5, and resistant G8 CR-EC isolates. PROVEAN analysis predicts that this deletion will have a deleterious effect on the enzymatic function of NfsA. The *nfsB* gene of these isolates was found to be unmutated, and unlike previous studies, no other previously reported mutations were found in the *nfsA* gene (Sandegren et al., 2008; Osei Sekyere, 2018; Zhang et al., 2018). Moreover, the promoter region of the *nfsA* gene was also found to be unchanged in these isolates. Therefore, some unknown mechanisms may cause elevated MIC in isolate G8 (128 µg/mL) compared to intermediate G4 and G5 isolates (64 µg/mL) (Table 3.1). A novel deleterious mutation (R203H) in the active site of NfsA in CR-EC isolate G16 might cause intermediate

susceptibility, as the substitutions (G66D, M75I, and V93A) found in NfsB were reported to be neutral (Sandegren et al., 2008; Zhang et al., 2018).

In the nitrofurantoin-resistant ESBL-EC isolate G10, a novel large deletion of 138 amino acid residues (del M-A (1-138)) at the N-terminal region of NfsA containing two of the three nucleotide-phosphate binding regions (11-15, and 128-131 aa) and FMN binding sites (39 and 67) was found. NfsA reduces nitrofurantoin antibiotic using nicotinamide adenine dinucleotide phosphate (NADPH), while FMN serves as a cofactor that mediates electron transfer to various electron acceptors from NADPH (Zenno et al., 1996; Kobori et al., 2001). As no other deleterious mutations were found in the *nfsA* and *nfsB* genes, the deletion of the functionally significant N-terminal region of NfsA may cause resistance in isolate G10. Nitrofurantoin resistance in ESBL-EC isolates G11 and G17 may be mediated by identical and novel deleterious SNPs in *nfsA* (P3G, T4P, and I5S) and *nfsB* (G153D, in one of the four nucleotide-phosphate binding regions) genes. An unknown mechanism may drive the nitrofurantoin resistance in G15 as this isolate only has previously described neutral mutations in the *nfsA* (I117T, K141E, and G187D) and *nfsB* (G66D and V93A) genes (Vervoort et al., 2014; Zhang et al., 2018).

3.3.3.6 Deleterious mutations in the regulatory genes of MDR efflux pumps may contribute to resistance

MDR efflux pumps can confer resistance to multiple antibiotic classes when overexpressed mainly due to mutations in the regulatory gene or promoter region of the transporter gene (Piddock, 2006; Sun et al., 2014; Blanco et al., 2016). Analysis of genes related to MDR efflux pumps in wastewater isolates identified several deleterious mutations in the regulatory genes of some of the efflux pumps.

Specifically, Breseq analysis identified various insertion-deletions (INDELs) and SNPs in the CR-EC isolates G4, G5, and G8 for the *acrR* gene, which encodes for a protein belonging to the TetR-family transcriptional regulators (TFTRs) involved in the regulation of the multidrug efflux pump (Wang et al., 2001; Ramos et al., 2005; Colclough et al., 2019). PROVEAN analysis found several deleterious non-synonymous SNPs (A2G, R3T, T5N, K6Q, Q7T, A9S, E11R, T12N, R13A, Q14P, H15T, I16H, L17P, D18R, A20G, L21S, and R22T) in these isolates (Table S3.4). Some of these deleterious

SNPs were found at the N-terminal conserved region (10-70 aa) of AcrR (T12N, R13A, Q14P, H15T, I16H, L17P, D18R, A20G, L21S, and R22T). Deleterious mutations in the conserved region of AcrR may have some adverse effects on the repression of multidrug efflux in XDR CR-EC isolates G4, G5, and G8.

A frameshift mutation due to deletion was observed in the *evgA* gene of the ESBL-EC isolate G10. EvgA is a DNA-binding transcriptional activator and a member of the two-component signal transduction system, EvgS/EvgA (Nishino & Yamaguchi, 2001). EvgA upon phosphorylation by EvgS regulates the expression of diverse genes conferring multidrug resistance to drug-hypersusceptible strains lacking the constitutive multidrug efflux pump gene *acrAB* (Ma et al., 1995; Nishino & Yamaguchi, 2001). The deletion of a nucleotide (i.e. A) from the 202 position introduced an early stop codon that results in the C-terminal deletion of 105 amino acids from the regulator, which removes the DNA-binding region (161-180 aa), the HTH luxR-type conserved-region (137-202 aa) and 14 residues (100-114 aa) of the response regulator receiver domain (4-114 aa). No deleterious mutations were found in the *evgS* gene (Table S3.4). As the G10 isolate has the *acrAB* genes, the functionally inactive EvgA may not have any adverse effect on the expression of a multidrug resistance phenotype.

Overall, the analysis of resistance mechanisms showed that most of the resistance against β-lactams, aminoglycosides, fluoroquinolones, and trimethoprim and sulfamethoxazole was caused by diverse combinations of previously defined clinically relevant resistant determinants. However, novel resistance mechanisms against nitrofurantoin antibiotics were predicted in wastewater isolates based on unique deleterious mutations in the nitroreductase encoding genes *nfsAB*. In addition, unknown resistance mechanisms against some antibiotics such as nitrofurantoin and cephamycin (i.e. cefoxitin) were also observed. The overexpressed MDR efflux due to deleterious mutations in regulatory genes might constitute novel multidrug resistance mechanisms in isolates lacking other resistance determinants.

3.3.4 Virulence genes content classified some isolates into extraintestinal pathotypes, while others remain unclassified

Virulence to cause intestinal or extraintestinal infections is mainly driven by various factors such as adhesins, siderophores or iron acquisition system, toxins, protectins/serum resistance (i.e. capsules, lipopolysaccharide components, and outer membrane proteins), invasins, and other putative virulence factors (Kaper et al., 2004; Croxen & Finlay, 2010; Sarowska et al., 2019). These virulence genes are often encoded on plasmids and other MGEs that facilitate mobilisation between different bacterial species (Croxen & Finlay, 2010; Sarowska et al., 2019). From the phylogenetic analysis described above, I found the relatedness of wastewater isolates with strains from human and non-human sources. Here, I sought to investigate whether these MDR and XDR wastewater isolates are also pathogenic to humans and animals. VirulenceFinder predicted multiple virulence genes from the draft genome of CR-EC and ESBL-EC isolates from wastewater (Table 3.2). From the virulence gene profile, the pathogenic potential of wastewater isolates can be determined.

Specifically, ESBL-EC isolates G11, G15, and G17 belonging to ST131 had multiple virulence factors, including adhesins (i.e. *fimH* encoding adhesion of type 1 fimbriae, *iha* encoding an IrgA homologue adhesin, and *papA_F43* encoding P fimbriae), siderophore/iron uptake (i.e. *fyuA* encoding a yersiniabactin receptor, *irp2* encoding yersiniabactin biosynthetic protein, and *chuA* for an outer membrane hemin receptor), protectins/serum resistance (i.e. *kpsE* encoding capsule polysaccharide export inner-membrane protein, *kpsMII_K5* for a group two capsular polysaccharide, *ompT* encoding an outer membrane protease T, and *traT* for complement resistance protein), and enterotoxin (i.e. *senB* encoding enterotoxin TieB protein) (Table 3.2). Isolates G11, G15, and G17 also had putative virulence factors such as bacteriocin (i.e. *usp* encoding for uropathogenic specific protein), fimbrial protein (i.e. *ycfV* encoding the major subunit of a putative chaperon-usher fimbria), *gad* encode for glutamate decarboxylase, and *terC* encoding tellurium ion resistance protein (Table 3.2). In addition, isolate G11 had other virulence factors such as siderophores (i.e. *iucC* encode for aerobactin synthetase and *iutA* encoding aerobactin receptor) and toxin (i.e. *sat* encode for secreted autotransported toxin) (Table 3.2). G15 also had an adhesin (i.e. *papC* encoding an outer membrane usher P fimbriae), a siderophores/iron uptake system

(i.e. *iucC, iutA,* and *sitA*) and toxins (i.e. *cnf1* encoding a cytotoxic necrotising factor 1 and *sat*) (Table 3.2). Although G11, G15, and G17 carried a considerable number of extraintestinal infection-related genes shared between different pathotypes (Riley, 2014; Sarowska et al., 2019), the presence of the uropathogenic *E. coli* (UPEC) predictor genes *chuA*, *ycfV,* and *fyuA* could classify these isolates as UPEC (Spurbeck et al., 2012).

Similar to ST131 isolates, the ESBL-EC isolate G10 belonging to the unique ST9586 type harboured several extraintestinal infection-related genes such as *fyuA*, *irp2, iucC*, *iutA* and *sitA* (siderophores), *ompT* and *traT* (serum resistance), *gad,* and *terC* (Table 3.2). In addition, G10 carried *ipfA* encoding for long polar fimbriae (adhesin), *iss* encoding increased serum survival protein (serum resistance), the *hlyF* gene encoding hemolysin F (pore-forming toxin), and *etsC* encoding putative type I secretion outer membrane protein (secretion system) (Table 3.2). G10 isolates could be classified as avian pathogenic *E. coli* (APEC)-like owing to the presence of *hlyF, iss, iutA,* and *ompT* predictor genes (Johnson et al., 2008).

CR-EC isolates G5 and G8 belonging to ST167 had several virulence factors such as *hra* encoding heat-resistant agglutinin (adhesin), *iss* and *traT* (serum resistance), *capU* encoding putative hexosyltransferase homolog, and *terC* (Table 3.2). In addition, G5 had the genes *fimH* (adhesin), *fyuA* and *irp2* (siderophore), and G8 carried *iucC* and *iutA* (siderophore) and *gad* genes (Table 3.2). The CR-EC isolate G4 of the ST1702 group carried virulence factors identical to isolate G5 (Table 3.2). Although isolates G4 and G5 had the APEC-related gene *iss* and the UPEC-associated gene *fyuA*, and G8 carried the APEC-related genes *iss* and *iutA*, these isolates could not be assigned to respective pathotypes due to the absence of a required number of other predictor genes (Johnson et al., 2008; Spurbeck et al., 2012). The CR-EC isolate G16 (ST410 type) had the genes *fimH*, *hra* and *ipfA* (adhesin), *astA* encoding heat-stable enterotoxin 1 (toxin), *gad,* and *terC* genes (Table 3.2). However, despite the fact that isolate G16 carried the enteroaggregative *E. coli* (EAEC) associated enterotoxin gene *astA*, it could not be classified into a specific diarrheagenic pathotype due to the lack of other genetic markers (Kimata et al., 2005; Fujioka et al., 2013).

In conclusion, pathotyping based on the virulence gene content indicated that all MDR ESBL-EC isolates from wastewater isolates belong to known extraintestinal pathotypes of humans and animals. However, despite carrying various extraintestinal and intestinal pathotypes-related genes, none of the XDR CR-EC isolates was classified into known pathotypes due to the lack of the required number of virulence markers. The lack of a sufficient number of virulence markers might also indicate that these *E. coli* isolates may not be able to cause symptomatic infections and, therefore, might not be sampled in diagnostic settings.

3.3.5 The potential of the wastewater isolates for the acquisition or transfer of antibiotic resistance and virulence genes

The identification of diverse genes conferring resistance and virulence led me to investigate whether the wastewater isolates have acquired or could transfer these genes. For this purpose, I examined the carriage of MGEs, such as plasmids that are considered the key vehicles for acquiring and disseminating antibiotic resistance and virulence genes among bacterial populations (Carattoli, 2013; Pilla & Tang, 2018; Rozwandowicz et al., 2018). PlasmidFinder identified several replicons suggestive of respective plasmids in XDR CR-EC and MDR ESBL-EC isolates (Table 3.2). In addition, manual inspection of contigs containing plasmid replicons indicated plasmid-mediated acquisition of resistance and virulence determinants in some wastewater isolates.

The XDR CR-EC isolate G4 harboured several plasmid replicons belonging to the colicinogenic (Col)-, IncF (multi-replicon)-, IncI- and IncQ-type (Table 3.2). The contig harbouring the IncQ2 plasmid replicon in isolate G4 also had the quinolone resistance gene *qnrs2*, suggesting the plasmid-mediated acquisition of this resistance gene (Table S3.5). My observation is consistent with previous studies that reported *qnrS2* gene dissemination by the IncQ2 plasmid (Han et al., 2012; Wen et al., 2016; Hayer et al., 2020; Piotrowska et al., 2020). Among the different Col-, IncF-, IncM-, and IncR-type plasmid replicons harboured by the XDR CR-EC isolate G5, the acquisition of the IncM2 plasmid may be associated with the carriage of the β-lactamase encoding gene $bla_{\text{TEM-1B}}$ (Table S3.5), as reported recently (Dor et al., 2020). The XDR CR-EC isolate G8 had several IncF- and IncI-type, and the G16 isolate harboured various Col-, IncF- and IncQ-type plasmid replicons (Table S3.5). In isolate G16, the IncQ1 plasmid might be related

to the acquisition of the sulfonamide resistance gene *sul2* (Table S3.5), as documented previously (Poirel et al., 2010; Yau et al., 2010; Rozwandowicz et al., 2018).

The MDR ESBL-EC isolates G10, G11, G15, and G17 harboured various Col- and IncF-type plasmid replicons in various combinations (Table 3.2). Isolates G11 and G17 also had an IncI-type plasmid replicon (Table 3.2). In isolate G10, the acquisition of the virulence genes *hylF* (toxin) and *ompT* (serum resistance) related to the APEC pathotype may be associated with the IncF-type plasmid (Table S3.5). This observation is consistent with previous studies that reported the dissemination of APEC-related virulence genes via the IncF plasmid (Johnoson & Nolan, 2009; Mellata et al., 2009; Olsen et al., 2012). The carriage of the enterotoxin encoding gene *senB* in isolates G11, G15, and G17 might be related to the presence of the Col156 plasmid (Table S3.5), which requires further confirmation. Manual inspection of the annotated genomes of G11, G15, and G17 identified that the *senB* gene was flanked by an insertion sequence ISSbo1 of the IS91 family, which was reported to be frequently associated with the dissemination of this virulence gene in *E. coli* (Garcillán-Barcia et al., 2002; Mbanga et al., 2021). In isolate G15, the carriage of $bla_{\text{TEM-1B}}$ was possibly associated with the acquisition of IncF-type plasmid (Table S3.5), according to previous reports (Kim et al., 2011; Khezri et al., 2020; Stephens et al., 2020).

Overall, I found evidence for the plasmid-mediated acquisition of several clinically relevant resistance and virulence genes in the XDR and MDR wastewater isolates. Identification of plasmid-borne resistance genes in some of the XDR CR-EC isolates of unknown pathotypes indicates that these isolates could serve as underexplored reservoirs of transferrable resistance genes.

3.3.6 Impact of mutations on bacterial survival and motility

Breseq analysis identified several mutations such as INDELs and SNPs in genes involved in diverse cellular functions in some of the wastewater isolates. Furthermore, the PROVEAN analysis found that some of these mutations have functional impacts on bacterial survival and motility.

Deletions and several non-synonymous SNPs were found in the *nfrA* gene, encoding an outer membrane receptor for the N4 bacteriophage adsorption, in isolates G4, G11, and G16 (Kiino et al., 1989). A deletion of a 15-bp sequence (1795-1809 nucleotide) that resulted in five amino acid deletions (del A-L (598-602)) from the NfrA in isolate G4 was found deleterious (Table S3.6). In isolate G11, a large deletion of 924 bp (1-924 nt), resulting in the deletion of 308 amino acids at the N-terminus of NfrA, was also found to be deleterious (Table S3.6). The deletion resulted in the loss of the signal peptide (1-27 aa), the signal peptidase cleavage sequence (25-27 aa), and the tetratricopeptide repeat 1 (TPR 1, 81-114 aa) domain in isolate G11. The loss of signal peptide and signal peptidase may prevent the translocation of the processed NfrA to the outer membrane (Kiino et al., 1989). As NfrA spans the membrane only once (Kiino et al., 1993), the impaired translocation may make this outer membrane receptor unavailable for N4 bacteriophage adsorption in isolate G11. The non-synonymous SNP, N645T, in G16 was found deleterious (Table S3.6). This mutation falls in one of the three TPR domains (TPR 2), used by phage N4 for irreversible interaction with NfrA (McPartland et al., 2009). Therefore, it may impede the interaction between the bacteriophage and the bacterial cell and provide a survival advantage by preventing phage-mediated lysis.

Frameshift mutations due to INDELs were identified in the *cirA* gene of isolates G4, G5, G8, and G10. Cir, encoded by *cirA*, serves as an outer membrane receptor for colicins IA and IB (Buchanan et al., 2007; Jakes et al., 2010). Colicins are bactericidal proteins produced by some *E. coli* strains in response to stress that kills competing *E. coli* by binding to the Cir receptor (Braun et al., 1994; Cascales et al., 2007). The deletion of two nucleotides (268-269/1992 nt) caused an early stop codon that resulted in the C-terminal deletion of 557 amino acids (106-663) that removed the transmembrane β barrels (164-663 aa) and 46 residues (106-152 aa) of the plug domain (44-152 aa) of the receptor in isolates G4, G5, and G8 (Table S3.6). Colicins bind to the two arginine residues in the extracellular β-barrel loops L7 and L8 of Cir that trigger conformational changes in the plug domain inside the barrel lumen to translocate colicins inside the target cell (Buchanan et al., 2007). Therefore, deletion of these regions may prevent the binding of colicin to its receptor in the G4, G5, and G8 isolates. Deleterious non-synonymous SNPs (S90Y, I91S, R92W, G93S, L94G, S97L, Y98H, T99P, L100D, I101S, L102R, V103R, and D104R) in the plug domain of the Cir receptor of isolates G4, G5, and G8 might also

impair the translocation of colicin inside the cell (Table S3.6). Insertion of a nucleotide between the nucleotide positions 475 and 476 (+A) introduced a premature stop codon that deleted 481 amino acids (182-663) from the C-terminal transmembrane β-barrels (164-663 aa) of Cir in G10 (Table S3.6). The deletion of most of the β-barrel residues along with deleterious non-synonymous SNPs in the rest of the fragments (W164M, T167Y, V168R, V170R, T172Y, T173H, I174H, Q175S, E176G, H177T, and G181R) may also prevent the binding of colicins to the Cir receptor in the G10 isolate. Therefore, deleterious mutations in the Cir receptor may offer a survival advantage by preventing the colicin-mediated killing of G4, G5, G8, and G10 isolates in competitive niches.

Various structural variations were also observed in genes associated with bacterial motility, such as *flhA* and *fliI* in isolates G4, G5, and G8, and *fliP* in isolate G10. FlhA is one of the six integral membrane proteins of the flagellar export apparatus required to form the rod structure (Minamino & Macnab 1999; Macnab, 2004). A large deletion of 87-bp (1-87 nt) resulting in the deletion of 29 amino acid residues (del M-L (1-29)) from the N-terminal of FlhA in isolates G4, G5, and G8 was predicted to have a deleterious effect (Table S3.6). Several non-synonymous mutations within one of the eight transmembrane regions (24-44 aa) of FlhA were also found in these isolates (I31S, L32N, S33L, M34A, M35A, V36M, and P38R). A previous study found that FlhA lacking the amino-terminal sequence before the first transmembrane span (1-22 aa) failed to export flagellar apparatus proteins resulting in an adverse effect on bacterial motility (McMurry et al., 2004). Therefore, the deletion of N-terminal amino acid residues and deleterious non-synonymous SNPs likely affect the motility of G4, G5, and G8 isolates.

Also related to motility is FliI, which is an ATPase that hydrolyses ATP to provide energy for the translocations of flagellar export substrates and is responsible for flagellar assembly across the cytoplasmic membrane (Fan & Macnab, 1996). A deletion of 1224 bp (151-1374 nt) resulting in the loss of 408 amino acid residues (del A-S (50-457)) from the C-terminal of FliI was predicted to be deleterious in isolates G4, G5, and G8 (Table S3.6). The deletion was caused by nucleotide substitutions (148-150, GCA->TGA) that introduced a stop codon at the 50 amino acid position (A50*). Deleterious non-synonymous substitutions were also found in isolates G4, G5, and G8 (L46N, P47L, L48C, and G49H) (Table S3.6). The deletion of the C-terminal region with ATPase

activity (381-450 aa) in isolates G4, G5, and G8 may prevent the hydrolysis of ATP required for the translocation of the flagellar export apparatus across the cytoplasmic membrane. The inactive *fliI* in isolates G4, G5, and G8 might prevent flagellar assembly across the cytoplasmic membrane and thus may have an adverse effect on their motility.

A single nucleotide deletion causing a frameshift mutation was observed in the *fliP* gene of isolate G10. FliP is one of the essential membrane components of the flagellar export apparatus (Fan et al., 1997; Minamino et al., 2004) required for the formation of the protein-conducting pore of the flagellar secretion apparatus (Ward et al., 2018). The deletion of a nucleotide C from nucleotide position 644 introduced an early stop codon that caused a C-terminal deletion of 22 amino acids (223-245), which deleted 13 residues (215-236 aa) from the conserved pore-forming transmembrane segment 4 (TM4) (Table S3.6). A previous study found that replacement of the invariant residue lysine from the 222 position of TM4 caused substantially reduced bacterial motility (Erhardt et al., 2017). I also found a replacement of lysine with an asparagine at the 222 position of TM4. Several other predicted deleterious non-synonymous SNPs, such as A215P, T216P, A218L, L219C, and F221L, were also found in TM4 (Table S3.6). The deletion of most of the amino acids from the TM4 of FliP, along with deleterious mutations in conserved regions, may impair the transport of flagellar assembly proteins and thus may have an adverse effect on the motility of the G10 isolate.

Overall, I found that some CR-EC and ESBL-EC isolates from wastewater evolved not only to become resistant to multiple antibiotics but also to prevent phage predation and colicin-mediated killing that may enable them to survive and proliferate in diverse competitive niches, which is concerning. In addition, I observed mutations that reduce motility in some CR-EC and ESBL-EC isolates that might adversely impact their ability to successfully colonise humans and animals host to cause infections (Josenhans & Suerbaum, 2002); however, this requires further investigation.

3.4 Conclusion

In this study, I found that XDR CR-EC and MDR ESBL-EC isolates from wastewater exhibit resistance against critically important antibiotics and are phylogenetically related to pandemic high-risk human-associated clones and to emerging human- and non-human-

associated clones. In addition, I identified that diverse known mechanisms underpin their resistance against most of the critically important antibiotics and found indications for novel resistance mechanisms against nitrofurantoin. Furthermore, I identified that the MDR ESBL-EC isolates were likely pathogenic and carried mobile virulence genes that can be transferred to other non-pathogenic bacteria, which is concerning. I also found that the XDR CR-EC isolates might be associated with asymptomatic infections due to the lack of the required number of virulence markers and, therefore, likely to be underexplored by clinical surveillance focusing on symptomatic infections. Nevertheless, with transferrable resistance genes and better fitness, these CR-EC isolates could serve as an important reservoir of critically important antibiotic resistance genes that warrant continuous monitoring of these likely non-pathogenic isolates. Overall, I found that wastewater-based surveillance has a clear potential to monitor resistance beyond clinical settings and can thus complement healthcare infection surveillance to inform infection control and prevention strategies to effectively track and control the emergence and spread of antibiotic resistance.

Chapter 4 Genomic analysis revealed the characteristics of extended-spectrum β-lactamases-producing and carbapenem-resistant *Klebsiella pneumoniae* and *Klebsiella variicola* strains isolated from urban wastewater

4.1 Introduction

Klebsiella pneumoniae and one of its close relatives, *Klebsiella variicola,* belong to the *Enterobacteriaceae* family and are usually harmless inhabitants of the gastrointestinal tract but capable of causing various infections in humans and animals (Jarvis et al., 1985; Podschun & Ullmann, 1998; Rosenblueth et al., 2004; Navon-Venezia et al., 2017; Rodríguez-Medina et al., 2019). In particular, these *Klebsiella* species are associated with various opportunistic infections such as urinary tract infections, wound or surgical site infections, pneumonia and bacteraemia, mainly in vulnerable individuals, such as neonates, immunocompromised people, the elderly, and hospitalised patients (Podschun & Ullmann, 1998; Navon-Venezia et al., 2017; Rodríguez-Medina et al., 2019). Additionally, *K. pneumoniae* and *K. variicola* have been found to be associated with severe community-acquired infections such as pyogenic liver abscesses, meningitis, and endophthalmitis that can occur in young and otherwise healthy individuals (Shon et al., 2013; Rodríguez-Medina et al., 2019). Several key virulence factors have been reported to be associated with hypervirulence, including specific capsular serotypes (i.e. K1, K2 K5, K20 and K57 in *K. pneumoniae* and K16 in *K. variicola*), overproduction of capsules or hypermucoidy due to the acquisition of *rmpA* or *rmpA2* genes, and the acquisition of siderophores such as aerobactin, salmochelin, and yersiniabactin, and the genotoxin colibactin (Shon et al., 2013; Rodríguez-Medina et al., 2019; Choby et al., 2020).

Various antibiotics such as β-lactams and aminoglycosides are used to effectively treat *Klebsiella* infections (Prince et al., 1997; Krause et al., 2016; Reyes et al., 2019). However, *Klebsiella* species resistant to multiple antibiotic classes, including last resort carbapenems and polymyxins, are increasing worldwide (WHO, 2014; Rodríguez-Medina et al., 2019), leaving very limited antibiotics for treatment. Infections caused by multidrug-resistant *Klebsiella* species are usually associated with treatment failure, extended hospitalisation, increased healthcare costs, and a likely increase in mortality

(Magiorakos et al., 2012; Iredell et al., 2016; Bassetti et al., 2018; Batalla-Bocaling et al., 2021). In Australia, *Klebsiella* species have been considered priority pathogens of clinical importance, and their resistance to clinically significant antibiotics has been monitored nationwide using the healthcare-based Antimicrobial Use and Resistance in Australia (AURA) surveillance system (AURA, 2016). The AURA surveillance has shown in recent years increasing resistance trends of *Klebsiella* species (mostly *K. pneumoniae* and a few *K. variicola*) isolated from healthcare- and community-acquired infections to commonly used agents such as third-generation cephalosporins, aminoglycosides, and fluoroquinolones (AURA, 2021). However, the resistance of *Klebsiella* species to carbapenem (meropenem) remains very rare in Australia (less than 0.6% of *Klebsiella* species in 2019), based on isolates from urine and blood cultures (AURA, 2021).

The major antibiotic resistance mechanism in *K. pneumoniae* and *K. variicola* is the production of different β-lactamases such as extended-spectrum β-lactamases (ESBL) that confer resistance to cephalosporins and monobactams, and carbapenemases that renders these organisms resistant to almost all available β-lactams, including carbapenems (Holt et al., 2015; Potter et al., 2018; Peirano & Pitout, 2019; Rodríguez-Medina et al., 2019; Wyres et al., 2020). The most frequently observed ESBLs in these *Klebsiella* species include sulfhydryl variable (SHV)-, temoniera (TEM)- and cefotaximase-Munich (CTX-M)-types, and carbapenemases include oxacillinase-48 (OXA-48)-, *K. pneumoniae* carbapenemases (KPC)-, New Delhi metallo-β-lactamase (NDM)-, imipenemase (IMP)-, and Verona integron-encoded metallo-β-lactamase (VIM)-types (Nordmann et al., 2011; Hennequin & Robin, 2016; Navon-Venezia et al., 2017; Rodríguez-Medina et al., 2019). Other clinically important resistance mechanisms include the production of aminoglycoside-modifying enzymes and ribosomal methyltransferases conferring resistance to aminoglycosides, and mutations at chromosomal target sites, such as topoisomerases, that confer resistance to quinolones (Shaw et al., 1993; Ramirez et al., 2013; Hooper & Jacoby, 2016; Wachino et al., 2020). Particularly concerning is the mobilisation of resistance determinants via transferrable genetic elements such as plasmids, resulting in the emergence and rapid local and global dissemination of multidrug-resistant *Klebsiella* species (Carattoli, 2013; Rozwandowicz et al., 2018). In addition, the global dissemination of multidrug-resistant *K. pneumoniae* is also reported to be mediated by several epidemiologically successful or high-risk clones

with a superior ability to cause multicontinental outbreaks, persistence and ease of transmission between different hosts, and enhanced pathogenicity or fitness (Woodford et al., 2011; Mathers et al., 2015; Wyres et al., 2020). Several multidrug-resistant high-risk clones have been reported based on the *K. pneumoniae* multilocus sequence typing (MLST) scheme (Diancourt et al., 2005), including the third-generation cephalosporin and/or carbapenem-resistant sequence type (ST) 258, ST11, ST512, ST340 of the clonal group (CG) 258, ST14 and ST15 belonging to CG15, and ST17 and ST20 of the CG20 (Woodford et al., 2011; Wyres et al., 2020). In addition, recent reports have indicated the emergence of carbapenem-resistant ST307 and ST147 as international high-risk antibiotic-resistant clones (Lowe et al., 2019; Peirano et al., 2020; Strydom et al., 2020). Other globally disseminated multidrug-resistant clones of *K. pneumoniae* include ST13, ST29, ST37, ST101, and ST268 (Marcade et al., 2013; Ludden et al., 2020; Wyres et al., 2020). The global prevalence of *K. variicola* has been masked by the misidentification as *K. pneumoniae* by conventional microbiological methods due to the overlapping phenotypic and biochemical properties (Berry et al., 2015; Long et al., 2017). However, the recently developed *K. variicola* MLST scheme has identified several ESBL- and carbapenemase-producing clones, mainly from human sources (Barrios-Camacho et al., 2019). Notably include ESBL-producing ST1, ST4, ST10, ST14, ST20, ST60, ST65, ST72, ST76-78, ST94, ST108, ST146, ST160, ST164, ST175-176, ST221-222, ST224-225, and ST270 clones, and carbapenemase-producing ST53, ST60, ST64, ST69, ST75-76, ST92-93, ST125, ST130, ST136, and ST183 clones (Barrios-Camacho et al., 2019).

In addition to their association with symptomatic infections, multidrug-resistant *Klebsiella* species have also been reported to be carried and spread asymptomatically by healthy individuals within the community (Podschun & Ullmann, 1998; Kader et al., 2007; Gómez et al., 2021). Furthermore, antibiotic-resistant *Klebsiella* species have also been isolated from diverse non-human sources such as animals, food products, and the environment (Wyres & Holt, 2018; Barrios-Camacho et al., 2019; Marques et al., 2019). Moreover, asymptomatic carriers and non-human sources have been proposed as potential reservoirs for the amplification and transmission of resistant *Klebsiella* species or genes across various niches (Martin et al., 2016; Gorrie et al., 2018; Wyres & Holt, 2018; Barrios-Camacho et al., 2019; Marques et al., 2019). Therefore, monitoring antibiotic-resistant *Klebsiella* species beyond clinical settings is crucial to effectively

track and control the emergence and dissemination of the increasing rate of resistance. However, current healthcare infection surveillance systems in Australia and many parts of the world are inadequate to monitor the emergence and spread of resistance in *Klebsiella* species beyond healthcare settings, such as asymptomatic carriers in the community and isolates from non-human sources. As wastewater collects bacteria from humans, including asymptomatic carriers, animals, and environmental sources, routine wastewater-based surveillance could be used to monitor the occurrence and diversity of resistant *Klebsiella* species that circulate in the community (Newton et al., 2015; Yan et al., 2018; Hassoun-Kheir et al., 2020; Savin et al., 2022).

In the previous study described in Chapter 2, I used wastewater-based surveillance to monitor the geospatial-temporal trends of antibiotic resistance in various priority pathogens in the Sydney community. From this surveillance, I sporadically identified several ESBL-producing and carbapenem-resistant *K. pneumoniae* and *K. variicola* isolates from untreated wastewater collected at six-time points between 2017 and 2019 from 25 wastewater treatment plants in Sydney. Here, I aimed to identify their antibiotic resistance pattern, phylogenetic relationship with known *Klebsiella* lineages, molecular mechanisms underpinning resistance, the potential for virulence, and their potential to acquire and disseminate resistance genes.

4.2 Methods

4.2.1 Isolation, identification, and antibiotic susceptibility testing of *Klebsiella* species

From a collection of ESBL-producing and carbapenem-resistant (CR) *K. pneumoniae* (KP) and *K. variicola* (KV) (n=30), six isolates exhibiting resistance to the highest number of antibiotics (see Table 4.1 for the list of antibiotics) that are critically important for human health (WHO, 2019b) were selected for this study. This collection was derived from influent wastewater during a surveillance of antibiotic resistance in Sydney, New South Wales, Australia, from 2017 to 2019, following the methods described in Chapter 2. In brief, serial dilutions (1:10 and 1:100) of wastewater samples were plated onto ChromID CARBA SMART and ESBL agar (bioMérieux, Marcy I'Etoile, France) and

green-blue colonies from CARBA SMART and green colonies from ESBL plates were precisely identified at the species level as *K. pneumoniae* or *K. variicola* using matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS; Biotyper version 3.1, Bruker Daltonics, Billerica, MA, USA).

The susceptibility of *K. pneumoniae* and *K. variicola* isolates to 18 different antibiotics incorporated in the AST-N246 card was determined using a VITEK 2 instrument (bioMérieux, Vitek-Australia Pty Ltd) in accordance with the manufacturer's instructions and the Clinical and Laboratory Standards Institute's guidelines (CLSI, 2018), as described in Chapter 2. The VITEK 2 advanced expert system™ (AES) software (version 8.0) confirmed the suspected green-blue colonies on the CARBA SMART agar as carbapenem-resistant based on the elevated meropenem resistance (i.e. MIC ≥ 4 µg/mL) following the CLSI guidelines (CLSI, 2018). Green colonies on ChromID ESBL agar, which were found to be highly specific for ESBL-producing *Enterobacteriaceae* (Blaak et al., 2015), were further confirmed as ESBL producers by VITEK 2 AES based on the MIC patterns of several β-lactam antibiotics (i.e. ampicillin, cefoxitin, cefazolin, ceftazidime, ceftriaxone, cefepime, and meropenem) and the β-lactams with β-lactamase inhibitors (i.e. amoxicillin/clavulanic acid, piperacillin/tazobactam, and ticarcillin/clavulanic acid) incorporated in the AST-N246 card. Resistance to fosfomycin was determined using 200µg fosfomycin disk containing 50µg glucose-6-phosphate (Mast Group Ltd., Bootle, UK) by the Kirby-Bauer disk diffusion technique (Bauer et al., 1966) following the CLSI guidelines (CLSI, 2018).

4.2.2 Genomic DNA extraction, whole-genome sequencing, assembly, and annotation

The selected strains of *K. pneumoniae* and *K. variicola* were revived from pure frozen stocks on horse blood agar (HBA) plates (bioMérieux). A single colony from the HBA plate was grown in LB broth at 37°C at an agitation speed of 120 rpm. From the overnight culture, genomic DNA was extracted using the Monarch® Genomic DNA Purification Kit (New England Biolabs, Australia) as recommended by the manufacturer. Whole-genome sequencing (WGS) was performed on the Illumina MiSeq sequencer at the Ramaciotti Centre for Genomics at UNSW Sydney using 250 bp paired-end reads. Raw sequencing reads quality was assessed using FastQC v.0.11.8 (Andrews, 2010). Paired-

end reads were trimmed with Trimmomatic v.0.38 (Bolger et al., 2014) to clip adapters and other Illumina-specific sequences, reads with a minimum length of 50 bases, and bases with a Phred quality score of less than 25 at the start and end of a read. Quality-filtered reads were assembled using the default parameters of the *de novo* genome assembler SPAdes v.3.15.0 (Bankevich et al., 2012), and the assembled genomes quality was evaluated using Quast v.5.0.2 (Gurevich et al., 2013). Genomes were annotated with Prokka v.1.14.5 with its default databases (Seemann, 2014).

4.2.3 Bioinformatic and phylogenetic analysis

The sequence types (ST) of the assembled *K. pneumoniae* genomes were assigned according to Diancourt et al. (2005) using the bacterial isolate genome sequence database (BIGSdb) tool built in the Institut Pasteur's *K. penumoniae* MLST website (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html). STs of the *K. variicola* genomes were determined using the bioinformatic tool available at http://mlstkv.insp.mx (Barrios-Camacho et al., 2019). Additionally, antibiotic resistance genes and resistance-associated chromosomal mutations were identified using ResFinder 4.1 (Bortolaia et al., 2020) and PointFinder incorporated in ResFinder (Zankari et al., 2017), respectively, using a minimum nucleotide identity of 90% and a minimum alignment coverage of 60%. Known and unknown mutations in the resistance gene predicted by PointFinder were verified by aligning the respective nucleotide and amino acid sequences of the isolate with the sequences of the sensitive isolates obtained from the NCBI RefSeq database (O'Leary et al., 2016) using clustal omega (Sievers & Higgins, 2014). The impact of observed mutations on protein function was predicted using the protein variation effect analyser v.1.1.3 (PROVEAN) tool (Choi & Chan, 2015). Virulence genes were investigated by the BIGSdb available at http://bigsdb.pasteur.fr/klebsiella. Plasmid replicons were predicted using PlasmidFinder 2.1 (Carattoli et al., 2014), using minimum nucleotide identity and alignment coverage of 90% and 60%, respectively. Contigs containing plasmid replicons predicted by PlasmidFinder were manually inspected for the presence of antibiotic resistance and virulence genes using the ResFinder and BIGSdb output, respectively. Antibiotic resistance or virulence genes are considered plasmid-encoded if located on a contig with a plasmid replicon.

Based on the *K. pneumoniae* MLST scheme (Diancourt et al., 2005), a maximum likelihood phylogeny of *Klebsiella* genomes was inferred from an alignment of concatenated MLST genes (*gapA-infB-mdh-pgi-phoE-rpoB-tonB*) produced using the default parameters of MAFFT v.7.407 (Katoh & Standley, 2013). A maximum likelihood phylogenetic tree was built using RAxML v.8.2.10 (Stamatakis et al., 2014) with the general time reversible (GTR)-Gamma model and 1,000 bootstrap iterations. A total of 125 *K. pneumoniae* isolates from across the world belonging to similar STs were downloaded from published studies **(**Wyres et al., 2016; Gomi et al., 2018; Gorrie et al., 2018; Ellington et al., 2019**)**, NCBI's Refseq and the Institut Pasteur's BIGSdb-Kp database (Table S4.2) to contextualise the wider relevance of wastewater isolates. These isolates were randomly selected from different countries, sources, and years to avoid selection bias and provide representative samples since there were too many genome sequences (n = 30,085) available in the Institut Pasteur's BIGSdb-Kp database (https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_isolates [accessed, 23/02/2023]) to perform phylogenetic analysis using the available high performance computing resources at UNSW Sydney. A total of 326 *K. variicola* isolates from around the globe belonging to various STs available in the *K. variicola* MLST database were also downloaded (https://mlstkv.insp.mx/? [accessed, 23/02/2023]) (Barrios-Camacho et al., 2019). All downloaded *K. pneumoniae* and *K. variicola* genomes were annotated using Prokka and antibiotic resistance genes were predicted using ResFinder. Pan-genomes were estimated for the study and global *K. pneumoniae* isolates with the same ST, and all selected *K. variicola* STs using the default parameters of Roary v.3.12.0 (Page et al., 2015). Maximum likelihood phylogenetic trees were constructed applying the GTR-Gamma model in RAxML from the alignments of concatenated core coding sequence produced using the -mafft option in Roary. The phylogenetic trees were visualised with relevant metadata using iTOL v.6.3 (Letunic & Bork, 2021).

4.3 Results and discussion

4.3.1 Antibiotic susceptibility pattern of *Klebsiella* isolates from wastewater

To understand whether *K. pneumoniae* and *K. variicola* isolates from wastewater have unusual or unique antibiotic resistance patterns, I assessed three CR-KP, 25 ESBL-KP,

and two CR-KV isolates for resistance against 19 common antibiotics (Supplementary Table S4.1).

Among the various resistance patterns observed in the *K. pneumoniae* isolates, a CR-KP isolate (G14) was found to be resistant to almost all antibiotics tested (Table S4.1), which is very rare in Australia based on data from human clinical samples (AURA, 2021; AGAR, 2019). Specifically, isolate G14 was resistant to all the highest priority critically important antibiotics such as third- and fourth-generation cephalosporins (i.e. ceftazidime, ceftriaxone, and cefepime), and fluoroquinolones (i.e. ciprofloxacin and norfloxacin) (Table 4.1). In addition, this isolate exhibited resistance to several high priority critically important antibiotics such as third-generation penicillin or aminopenicillin (i.e. ampicillin), carbapenem (i.e. meropenem), penicillins with β-lactamase inhibitors (i.e. amoxicillin/clavulanic acid and piperacillin/tazobactam), and aminoglycoside (i.e. gentamicin) (Table 4.1). Furthermore, the G14 isolate showed resistance to all highly important antibiotics tested such as first- and second-generation cephalosporins (i.e. cefazolin and cefoxitin, respectively), dihydrofolate reductase inhibitor (i.e. trimethoprim), and dihydrofolate reductase inhibitor and sulfonamide combinations (i.e. trimethoprim-sulfamethoxazole) (Table 4.1). Resistance to important antibiotics such as the nitrofurantoin derivative (i.e. nitrofurantoin) and uncategorised ticarcillin/clavulanic acid was also exhibited by isolate G14 (Table 4.1). This isolate was sensitive to an aminoglycoside (i.e. amikacin) and the phosphonic acid derivative, such as fosfomycin (Table 4.1). The isolate G14 can thus be considered extensively drug-resistant (XDR) (Magiorakos et al., 2012), as it has only gentamicin and fosfomycin as a last resort treatment.

Among the diverse resistance pattern observed for ESBL-KP isolates (Table S4.1), I chose three isolates (i.e. G6, G7, and G9) that exhibited resistance against the highest number of critically important, highly important, and important antibiotics for human health for further analysis (WHO, 2019b) (Table 4.1). In particular, ESBL-KP isolates G6, G7, and G9 are considered multidrug-resistant (MDR) as they were resistant to at least three classes of antibiotics (Magiorakos et al., 2012). These isolates exhibited resistance to ceftazidime and ceftriaxone (highest priority critically important), ampicillin (high priority critically important), cefazolin, trimethoprim and trimethoprim-

sulfamethoxazole (highly important), and nitrofurantoin (important) (Table 4.1). However, isolates G6, G7, and G9 were found to be susceptible to norfloxacin (highest priority critically important), meropenem, amikacin and fosfomycin (high priority critically important), and cefoxitin (highly important) (Table 4.1). In addition, varying resistant patterns were observed against the rest of the antibiotics tested in isolates G6, G7, and G9 (Table 4.1).

Among the two carbapenem-resistant *K. variicola* isolates identified, one (i.e. G13) exhibited resistance against all but one or two of the antibiotic classes tested and thus can be defined as XDR (Magiorakos et al., 2012). Specifically, isolate G13 exhibited resistance to all tested β-lactam antibiotics, penicillins with β-lactamase inhibitors and ciprofloxacin (highest priority critically important), gentamicin (high priority critically important), trimethoprim and trimethoprim-sulfamethoxazole (highly important), and nitrofurantoin (important) (Table 4.1). However, isolate G13 was found to be sensitive to amikacin and fosfomycin (high priority critically important) (Table 4.1). To the best of my knowledge, XDR CR-KV has never been reported in Australia. Another CR-KV isolate (i.e. G12) was defined as MDR owing to its resistance to at least three classes of antibiotics (Magiorakos et al., 2012). Specifically, the CR-KV isolate G12 exhibited resistance to all tested β-lactam antibiotics, except carbapenems against which it showed intermediate susceptibility (Table 4.1). In addition, this isolate was resistant to all tested penicillins with β-lactamase inhibitors and intermediately susceptible or susceptible to the rest of the tested antibiotics (Table 4.1).

XDR and MDR *Klebsiella* species are a significant threat to public health because these bacteria often cause infections that are difficult to treat and sometimes even cause the death of infected people (Giske et al., 2008; Magiorakos et al., 2012; Bhatt et al., 2015; Rodríguez-Medina et al., 2019; Batalla-Bocaling et al., 2021). Therefore, it is imperative to understand the underlying resistance mechanisms and track the possible transmission sources of resistant bacteria and resistance genes to guide the appropriate treatment and interventions to control the emergence and dissemination of such XDR and MDR bacteria.

Based on the above information, CR-KP isolate G14, ESBL-KP isolates G6, G7 and G9, and CR-KV isolates G12 and G13 were selected for whole-genome sequencing to understand their relatedness with known *Klebsiella* lineages, the genetic mechanisms underpinning resistance and virulence, and their potential for acquisition and spread of antibiotic resistance and virulence-encoding genes.

Table 4.1 Antibiotic susceptibility pattern of the selected carbapenem-resistant (CR) and extended-spectrum β-lactamases (ESBL)-producing *K. pneumoniae* (KP) and *K. variicola* (KV) species isolated from wastewater.

Isolates	Year of collection	Phenotypes	Antibiotic Classes																		
			β-lactams							Penicillins with β-lactamase inhibitors			Aminoglycosides			Fluoroquinolones		Dihydrofolate reductase inhibitors	Combinations	Nitrofuran derivatives	Phosphonic acid derivatives
			Aminopenicillin	Cephamycin	Cephalosporins				Carbapenem												
			Ampicillin	Cefoxitin	Cefazolin	Ceftazidime	Ceftriaxone	Cefepime	Meropenem	Amoxicillin/clavulanic acid	Piperacillin/tazobactam	Ticarcillin/clavulanic acid	Gentamicin	Tobramycin	Amikacin	Ciprofloxacin	Norfloxacin	Trimethoprim	Trimethoprim-Sulfamethoxazole	Nitrofurantoin	Fosfomycin
G14	2018	CR-KP	**R** (>=32*)	**R** (>=64)	**R** (>=64)	**R** (>=64)	**R** (16)	**R** (16)	**R** (>=16)	**R** (>=32)	**R** (64)	**R** (>=128)	**R** (>=16)	**I** (8)	**S** (<=2)	**R** (>=4)	**R** (>=16)	**R** (4)	**R** (80)	**R** (256)	**S** (17 mm#)
G6	2017	ESBL-KP	**R** (>=32)	**S** (<=4)	**R** (>=64)	**R** (16)	**R** (>=64)	**R** (>=64)	**S** (<=0.25)	**R** (>=32)	**I** (32)	**R** (>=128)	**R** (>=16)	**R** (>=16)	**S** (<=2)	**R** (2)	**S** (2)	**R** (>=16)	**R** (>=320)	**R** (128)	**S** (16 mm)
G7	2018	ESBL-KP	**R** (>=32)	**S** (<=4)	**R** (>=64)	**R** (>=64)	**R** (>=64)	**R** (32)	**S** (<=0.25)	**R** (>=32)	**I** (32)	**R** (>=128)	**R** (>=16)	**R** (>=16)	**S** (4)	**R** (2)	**S** (4)	**R** (>=16)	**R** (>=320)	**R** (128)	**S** (20 mm)
G9	2018	ESBL-KP	**R** (>=32)	**S** (<=4)	**R** (>=64)	**R** (16)	**R** (>=64)	**S** (2)	**S** (<=0.25)	**I** (16)	**S** (16)	**I** (64)	**S** (<=1)	**I** (8)	**S** (<=2)	**S** (<=0.25)	**S** (2)	**R** (>=16)	**R** (>=320)	**R** (128)	**S** (19 mm)
G13	2018	CR-KV	**R** (>=32)	**R** (>=64)	**R** (>=64)	**R** (>=64)	**R** (>=64)	**R** (16)	**R** (>=16)	**R** (>=32)	**R** (>=128)	**R** (>=128)	**R** (>=16)	**I** (8)	**S** (<=2)	**R** (>=4)	**I** (8)	**R** (8)	**R** (80)	**R** (128)	**S** (16 mm)
G12	2018	CR-KV	**R** (>=32)	**R** (>=64)	**R** (>=64)	**R** (16)	**R** (16)	**R** (16)	**I** (2)	**R** (>=32)	**R** (>=128)	**R** (>=128)	**S** (4)	**I** (8)	**S** (<=2)	**S** (<=0.25)	**S** (2)	**S** (<=0.5)	**S** (<=20)	**S** (<=16)	**S** (18 mm)
Categories of important antibiotics for human medicine (WHO, 2019b)			Critically important (High priority)	Highly important	Highly important	Critically important (Highest priority)	Critically important (Highest priority)	Critically important (Highest priority)	Critically important (High priority)	Critically important (High priority)	Critically important (High priority)	Not available	Critically important (High priority)	Critically important (High priority)	Critically important (High priority)	Critically important (Highest priority)	Critically important (Highest priority)	Highly important	Highly important	Important	Critically important (High priority)

Acronym: R = resistant, **I** = intermediate, and **S** = sensitive.
* MIC (µg/mL).
# Inhibition zone diameter (nearest whole millimetre (mm)).

4.3.2 Genome and phylogenetic analysis

A summary of the genomic features of the *K. pneumoniae* and *K. variicola* isolates from wastewater is shown in Table S4.3. Assembly of the quality-filtered paired-end reads yielded 46 to 224 contigs with an N50 between 100 and 353 kilobase pairs (kbp). The total length of the assembled genomes ranges from around 5.52 to 6.05 megabase pairs (Mbp), with G + C content between 56.55 and 57.34% (Table S4.3). The assembled genomes were predicted to have between 5,240 and 5,830 genes and 5,179 and 5,763 coding sequences (CDS) (Table S4.3).

Phylogenetic analysis of wastewater isolates based on the *K. pneumoniae* MLST scheme (Diancourt et al., 2005) revealed diverse sequence types (ST) (Figure 4.1). For instance, the *K. pneumoniae* isolates G6, G7, G9, and G14 belonged to ST13, ST353, ST268, and ST2791, respectively (Figure 4.1). The *K. variicola* isolate G13 was assigned to ST3955 and G12 to a new ST termed *8cb6 (Figure 4.1). Previous studies found that STs assigned to *K. variicola* isolates based on the *K. pneumoniae* MLST scheme were shared with *K. pneumoniae* isolates, making it difficult to establish the genetic relationship among *K. varricola* isolates (Potter et al., 2018; Barrios-Camacho et al., 2019). Therefore, a separate MLST scheme was developed to follow the evolution of *K. varricola* isolates exclusively (http://mlstkv.insp.mx/) (Barrios-Camacho et al., 2019). This MLST scheme classified *K. varricola* isolates G13 into ST101 and G12 into a likely new ST, as discussed below (Figure 4.1). The *K. pneumoniae* isolates were not assigned an ST based on this scheme (Figure 4.1). For the subsequent phylogenetic analysis, *K. pneumoniae* and *K. variicola* isolates from wastewater were compared with global isolates belonging to related STs determined by the respective MLST scheme.

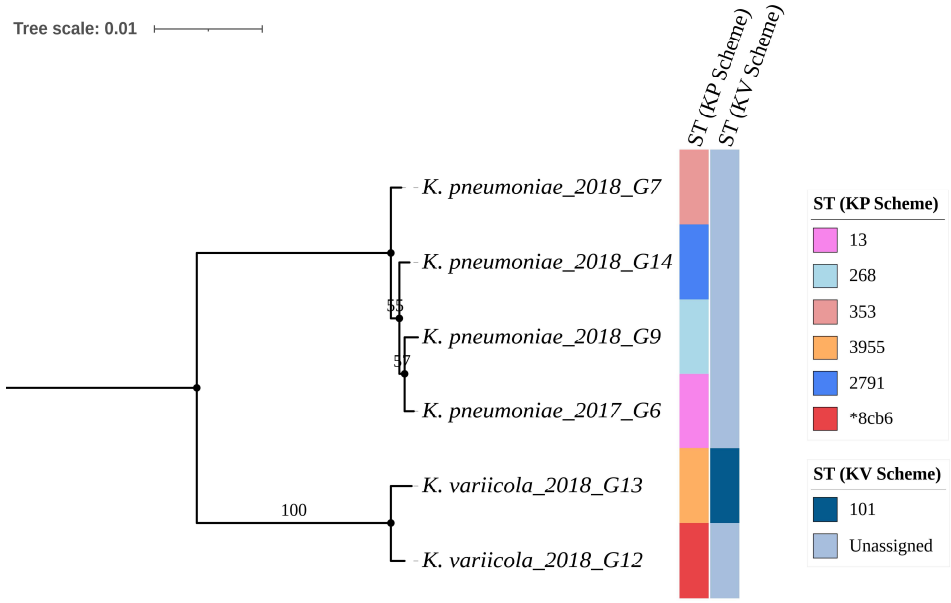


Figure 4.1 Maximum likelihood phylogenetic tree of *Klebsiella* isolates from wastewater (n = 6). The tree was inferred from the alignment of concatenated gene alleles (*gapA-infB-mdh-pgi-phoE-rpoB-tonB*) based on the *K. pneumoniae* MLST scheme (Diancourt et al., 2005) extracted from the genome assembly applying the GTR-Gamma model with 1,000 bootstraps in RAxML and is midpoint rooted. The tree nodes are labelled with species, year of isolation, and ID of the isolates. The coloured strips indicate different sequence types (ST) identified based on the *K. pneumoniae* (KP) and *K. varricola* (KV) MSLT scheme (http://mlstkv.insp.mx/) (Barrios-Camacho et al., 2019).

4.3.2.1 First detection of ST2791 and ST353 *K. pneumoniae* isolates of likely human origin in Australia

Around 4,280 core CDS (Supplementary Figure S4.1) were identified by Roary analysis in the XDR CR-KP isolate G14 and the closely related MDR ESBL-KP isolate G7 from wastewater and other global isolates belonging to the ST2791 (n = 2) and ST353 (n = 7) clones. Phylogenetic analysis based on the core CDS demonstrated two distinct clonal groups of the ST2791 and ST353 isolates, mainly of human origin (Figure 4.2). It was evident from the phylogeny that the ST2791 clone has emerged in recent years in Italy (2013), and ST353 isolates have been intermittently detected from around the world since 2005 (Colombia_2005_NZ_ NCLV00000000.1) (Figure 4.2). As far as I am aware, *K. pneumoniae* belonging to ST2791 and ST353 have never been reported in Australia.

From the phylogenetic analysis (Figure 4.2), it was also evident that the XDR ST2791 isolate G14 from wastewater in 2018 was very similar to MDR-type, human clinical

isolates from Italy in 2013 (GenBank accession number, NZ_UKNP00000000.1) and from a Japanese municipal wastewater treatment plant in 2015 (BioSample accession number, SAMD00092905) (Figure 4.2). However, isolate G14 was found to encode more resistance genes against β-lactams, including carbapenem (*bla*$_{\text{IMP-4}}$, *bla*$_{\text{OXA-48}}$, *bla*$_{\text{SHV-187}}$, and *bla*$_{\text{TEM-1B}}$), aminoglycosides (*aac(6')-Ib-cr, aac(3)-IId,* and *aac(6')-Ib3*), fluoroquinolones (*aac(6')-lb-cr, qnrA1,* and *qnrB2*), sulfonamides (*sul1*), and fosfomycin (*fosA*) compared to closely related isolates (Figure 4.2).

The MDR ST353 isolate G7 from wastewater in 2018 was related to an MDR-type, human clinical isolates from Colombia found in 2005 (NZ_NCLV00000000.1) carrying resistance genes against β-lactams, including carbapenem (*bla*$_{\text{KPC-2}}$, *bla*$_{\text{SHV-187}}$, and *bla*$_{\text{TEM-1B}}$), aminoglycosides (*aadA2*, *aadA10*, *aph(3')-Ia, aph(3')-Ib, aph(6)-ld, aac(3)-IIa,* and *aac(6')-lan*), fluoroquinolones (*oqxAB*), sulfonamides (*sul1*), trimethoprim (*dfrA1* and *dfrA12*), and fosfomycin (*fosA6*) (Figure 4.2). However, the G7 isolate has fewer resistance genes against β-lactams (*bla*$_{\text{OXA-1}}$, *bla*$_{\text{CTX-M-15}}$, and *bla*$_{\text{SHV-187}}$), aminoglycosides (*aph(3')-Ib, aph(6)-ld,* and *aac(3)-IId*), fluoroquinolones (*oqxAB* and *qnrB2*), sulfonamides (*sul2*), trimethoprim (*dfrA17*), and fosfomycin (*fosA6*) than the related Colombian isolate (Figure 4.2). The detection of carbapenemase-producing XDR clones within the ST2791 cluster and the MDR ST353 clones underscores the potential of wastewater testing to monitor the emerging *K. pneumoniae* clone in the community.

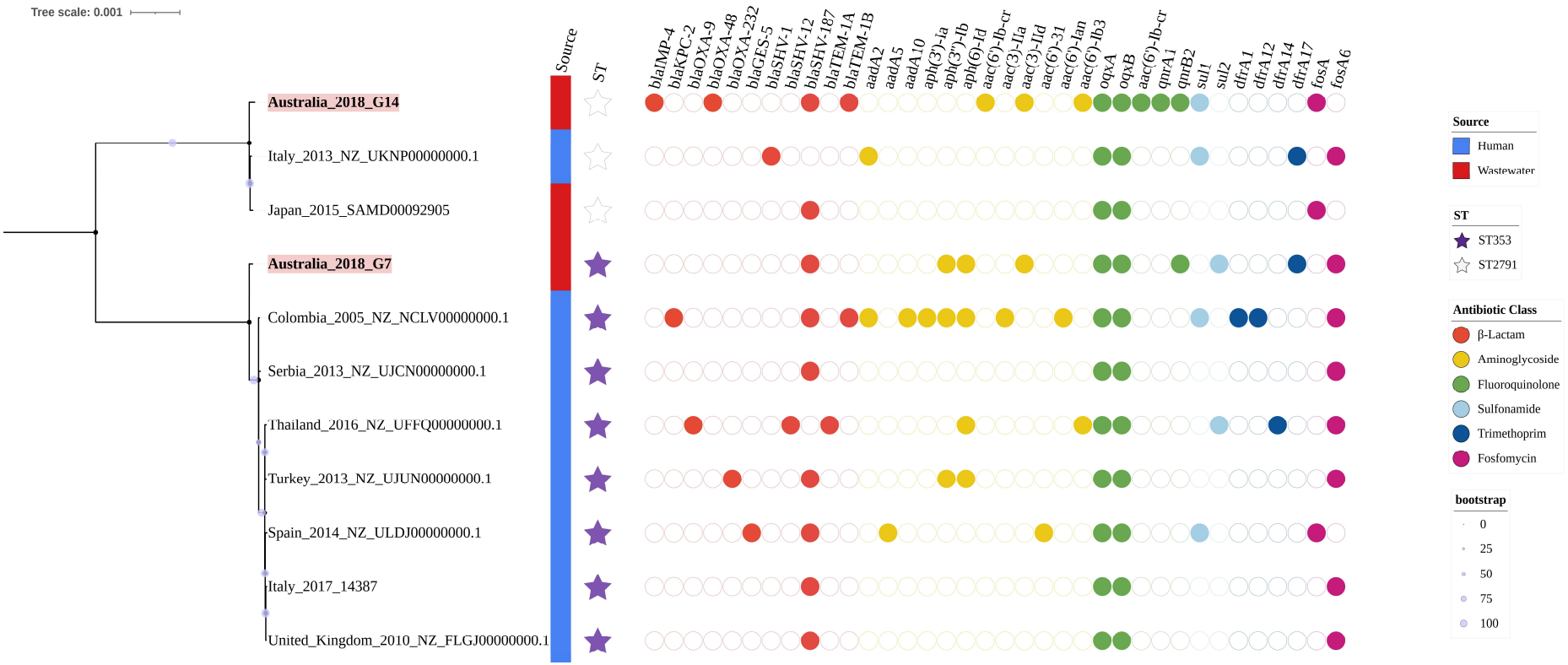


Figure 4.2 The maximum likelihood phylogeny of ST353 and related ST2791 *K. pneumoniae* isolates (n = 11) was inferred from the alignment of the concatenated core CDS (n = 4,280) determined by Roary and RAxML using the GTR-Gamma model and 100 bootstrap iterations. The tree is midpoint rooted, and the tree nodes are labelled with the origin, year of isolation, and NCBI GenBank accession number or the BIGSdb-Kp ID of the isolates provided by the Institut Pasteur (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html). The wastewater isolates are in bold and highlighted in pink. The source of the isolates, sequence types (ST), and acquired resistance genes against clinically important antibiotic classes are annotated on the coloured strips according to the given key.

4.3.2.2 A *K. pneumoniae* isolate from wastewater is related to globally disseminated ST13 strains from human sources

The Roary analysis identified around 3,962 core CDS (Figure S4.2) in the MDR ESBL-KP isolate G6 from wastewater and the randomly selected global isolates (n = 50) belonging to ST13 clones. Phylogenetic analysis based on the core CDS demonstrated multiple clades of ST13 isolates with no distinct geographical and temporal clustering (Figure 4.3). In addition, it was apparent from the phylogenetic tree that ST13 isolates have been around the world since 1999 (United_States_1999_NZ_SUOH00000000.1) and in Australia since 2009 (Australia_2009_SAMN06112128) (Figure 4.3).

It was also evident that the MDR isolate G6 from wastewater in 2017 was phylogenetically closely related to an MDR-type Australian isolate from human urine samples in 2013 (SAMEA3357259) carrying resistance genes against β-lactams ($bla_{\text{SHV-101}}$), fluoroquinolones (*oqxAB*), and fosfomycin (*fosA6*) (Figure 4.3). Isolate G6 possesses more resistance genes against β-lactams ($bla_{\text{OXA-1}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{SHV-101}}$, and $bla_{\text{TEM-1B}}$), aminoglycosides (*aph(3')-Ib, aph(6)-Id, aac(6')-Ib-cr,* and *aac(3)-IIa*), fluoroquinolones (*oqxAB, aac(6')-Ib-cr,* and *qnrB1*), sulfonamides (*sul2*), trimethoprim (*dfrA14*), and fosfomycin (*fosA6*) compared to the closely clustered Australian isolate (Figure 4.3).

ST13 has been described as an internationally disseminated MDR clone of *K. pneumoniae* due to its isolation from around the world, mainly in humans and various non-human sources (Marcade et al., 2013; Touati & Mairi, 2020; Franklin-Alming et al., 2021; Klaper et al., 2021; Shnaiderman-Torban et al., 2021). For instance, nosocomial (i.e. healthcare-associated) outbreaks caused by β-lactamases producing the MDR ST13 clone have been documented in France (Marcade et al., 2013), Spain (Diestra et al., 2011), and Ghana (Eibach et al., 2016). In addition, MDR ST13 strains carrying β-lactamase genes have also been isolated in non-human sources such as in livestock from Germany (Klaper et al., 2021), poultry from Norway (Franklin-Alming et al., 2021), and companion animals from Israel (Shnaiderman-Torban et al., 2021). Alarmingly, the carbapenemase-producing ST13 clone has been identified in recent years in human clinical samples from several countries, such as OXA-48-producing strains from Algeria (Mairi et al., 2019; Touati & Mairi, 2020), Finland (Österblad et al., 2012), Ireland (Wrenn et al., 2014) and Russia (Fursova et al., 2020); KPC-producing isolates from Colombia, the United States,

France and the United Kingdom (Figure 4.3); and NDM-producing isolates from China (Figure 4.3). Furthermore, the OXA-48-type carbapenemase-producing ST13 clone has been detected in a range of non-human sources from Algeria, including wild and companion animals, livestock, food, and the environment (Touati & Mairi, 2020); and NDM-producing isolates in poultry from China (Figure 4.3). Therefore, continuous monitoring of MDR ST13 clones in humans and a diverse range of non-human sources is required to track and control the emergence and spread of this clone.

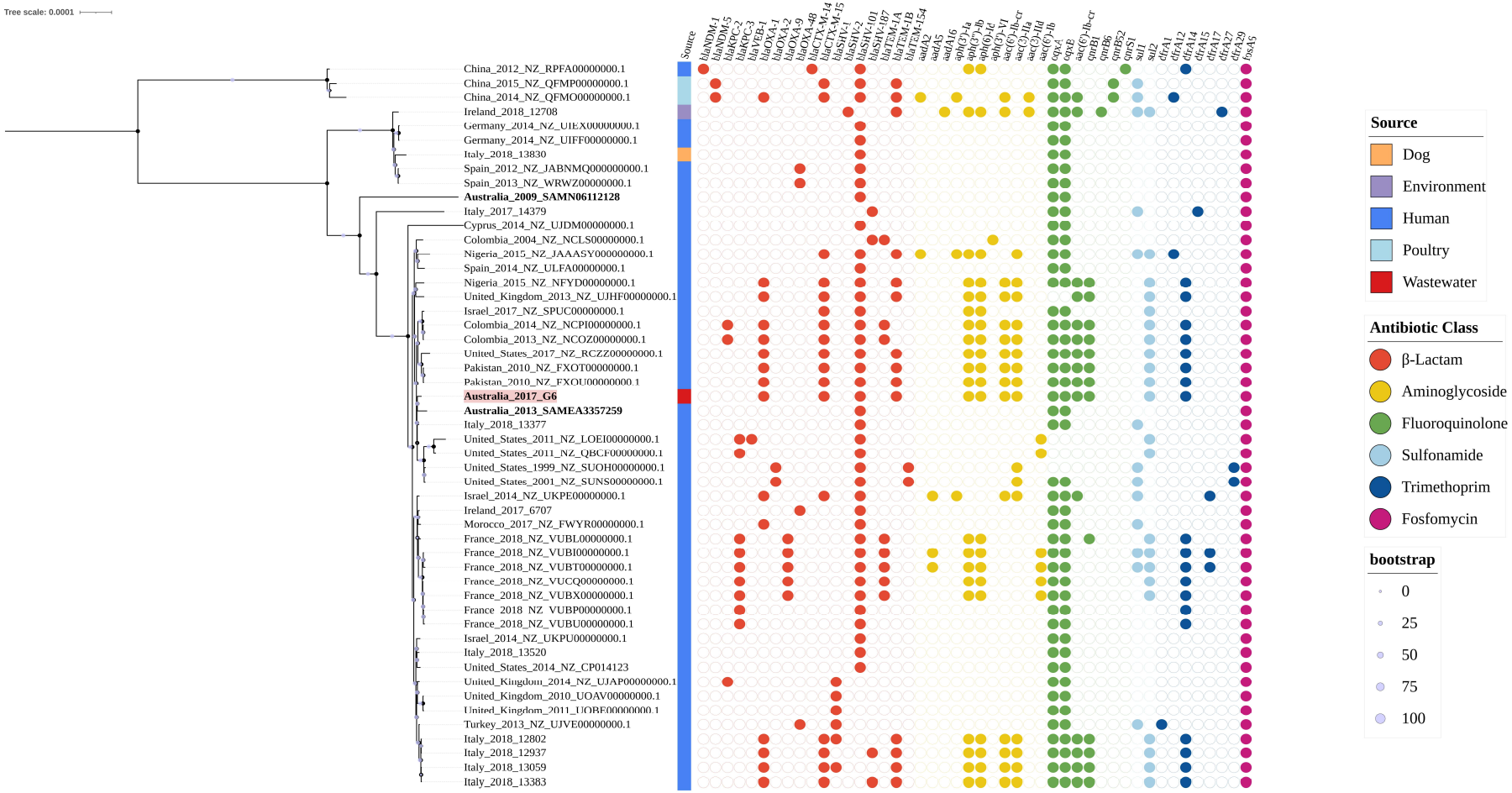


Figure 4.3 Maximum likelihood phylogeny of 51 ST13 *K. penumoniae* isolates inferred from the alignment of the concatenated core CDS (n = 3,962) determined by Roary and RAxML using the GTR-Gamma model and 100 bootstrap iterations. The tree is midpoint rooted, and the tree nodes are labelled with the origin, year of isolation, and NCBI GenBank accession number or the BIGSdb-Kp ID of the isolates provided by the Institut Pasteur (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html). The wastewater isolate belonging to ST13 is in bold and highlighted in pink, and the remaining Australian isolates are in bold. The source of the isolates and acquired resistance genes against different clinically important antibiotic classes are annotated on the coloured strips according to the given key.

4.3.2.3 A *K. pneumoniae* isolate from wastewater is related to ST268 clone of human origin

A total of 4,169 core CDS (Figure S4.3) were identified by Roary analysis in the MDR ESBL-KP isolate G9 from wastewater and randomly selected global isolates (n = 66) belonging to the ST268 clone. The phylogenetic tree based on the core CDS demonstrated multiple clades of ST268 isolated sporadically from around the globe in humans and environments since 2006 (Italy_2006_CCHC00000000.1) and from Australia since 2013 (SAMEA3357034, SAMEA3357048, and SAMEA3357114) (Figure 4.4). The clustering of isolates was irrespective of the isolates' source, collection year, and geographic location (Figure 4.4).

The ST268 isolate G9 from wastewater was found to be closely related to an MDR-type, carbapenemase-producing human isolate from Thailand found in 2016 (NZ_UFES00000000.1), which carried resistance genes against β-lactams ($bla_{\text{OXA-232}}$ and $bla_{\text{SHV-11}}$), fluoroquinolones (*oqxAB*), and fosfomycin (*fosA5*). However, the ESBL-producing isolate G9 carried more resistance genes against multiple antibiotic classes such as β-lactams ($bla_{\text{OXA-1}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{SHV-11}}$, and $bla_{\text{TEM-1B}}$), aminoglycosides (*aadA2* and *aac(6')-Ib-cr*), fluoroquinolones (*oqxAB,* and *aac(6')-Ib-cr*), sulfonamides (*sul1*), trimethoprim (*dfrA12*), and fosfomycin (*fosA5*) than the closely clustered carbapenemase-producing isolate from Thailand (Figure 4.4).

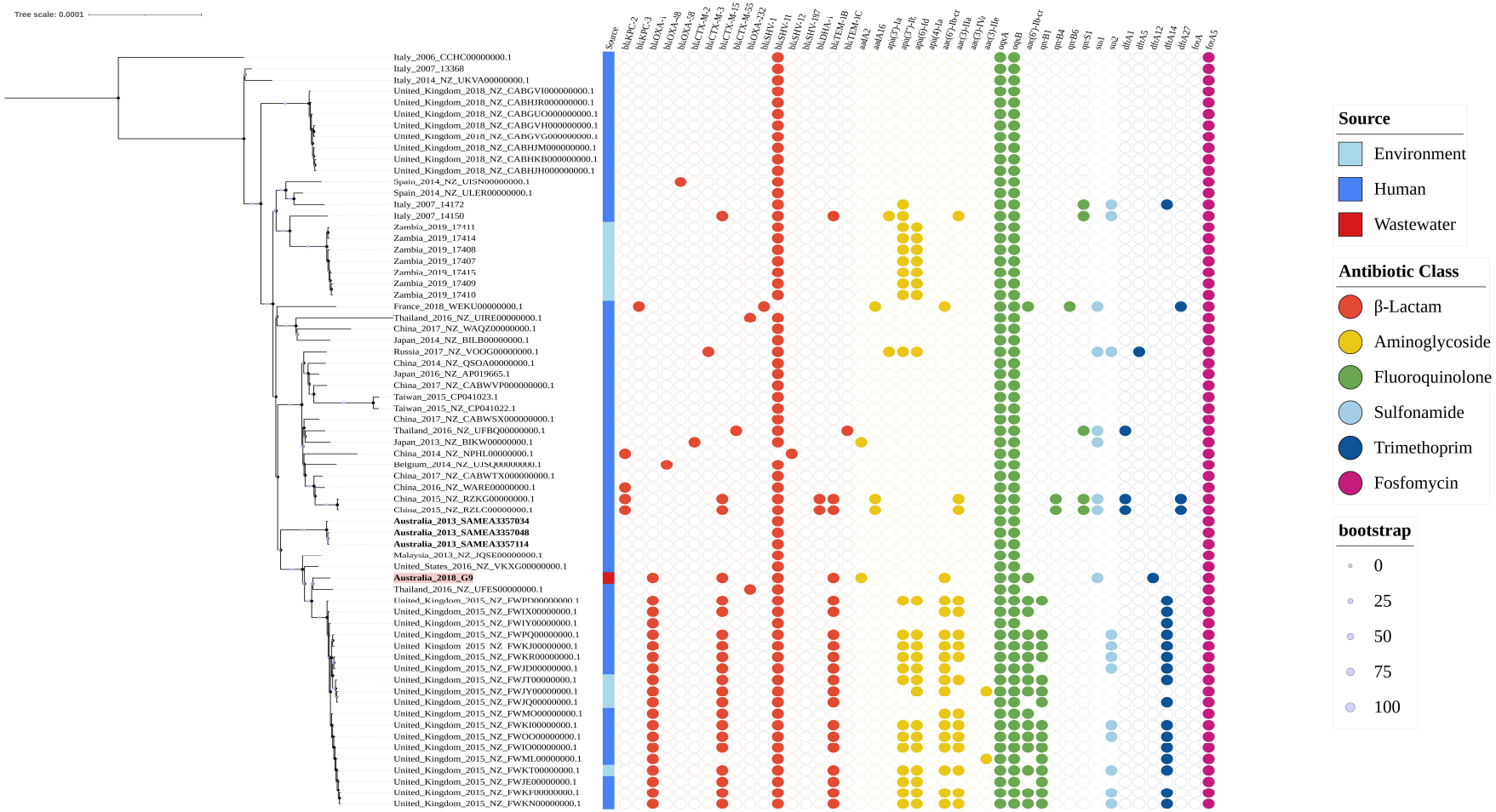


Figure 4.4 Maximum likelihood phylogeny of 67 ST268 *K. penumoniae* isolates inferred from the alignment of the concatenated core CDS (n = 4,169) determined by Roary and RAxML using the GTR-Gamma model and 100 bootstrap iterations. The tree is midpoint rooted, and the tree nodes are labelled with the origin, year of isolation, and NCBI GenBank accession number or the BIGSdb-Kp ID of the isolates provided by the Institut Pasteur (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html). The wastewater isolate belonging to ST268 is in bold and highlighted in pink, and the remaining Australian isolates are in bold. The source of the isolates and acquired resistance genes against different clinically important antibiotic classes are annotated on the coloured strips according to the given key.

ST268 isolates have been sporadically detected throughout the world in severe human infections, such as liver abscess, bacteraemia, pneumonia, urinary tract and invasive infections (Ko et al., 2010; Ito et al., 2015; Hirai et al., 2016; Zhang et al., 2016). Concerningly, several healthcare-based studies reported the association of the MDR, ESBL-producing ST268 clone with severe infections such as bloodstream infections in Taiwan (Yan et al., 2015), Denmark (Hansen et al., 2020) and Japan (Harada et al., 2019); liver abscess in China (Zhang et al., 2016); pneumonia in Japan (Ito et al., 2015), China (Xu et al., 2018) and Russia (Fursova et al., 2021); urinary tract infections in Korea (Ko et al., 2010), Japan (Kakuta et al., 2020), and Russia (Fursova et al., 2021). Recently, the possible patient-environment transmission of the CTX-M-type ESBL-producing ST268 clone has been documented in the haematology ward of a hospital in England (Ludden et al., 2020). Alarmingly, in recent years carbapenemase-producing ST268 strains have been isolated from patients with bloodstream infections in China (Shen et al., 2020), and bloodstream and urinary tract infections in Iran (Kiaei et al., 2019; Aslani et al., 2021). Furthermore, the ST268 clone was also identified in German municipal wastewater and surface waters (Savin et al., 2022), suggesting the likely dissemination of this clone in the community and the environment, respectively. Detection of MDR ST268 isolate G9 in wastewater underscores the continuous monitoring of this virulent clone within and beyond clinical settings to track and control its emergence and spread.

4.3.2.4 *K. variicola* isolates from wastewater belong to a new ST and emerging ST101 clones of human origin

The Roary analysis identified around 3,336 core CDS (Figure S4.4) in the XDR CR-KV isolate G13 and the MDR CR-KV isolate G12 from wastewater and the randomly selected global isolates (n = 326) belonging to various STs (n = 209). Phylogenetic analysis based on the core CDS indicated multiple clades of *K. variicola* isolated from around the globe from human and non-human sources since 1952 (Denmark_1952_NZ_USZY00000000.1) and from Australia since 2001 (NZ_LR130539.1 and SAMEA882280) (Figure S4.5). The clustering of isolates was independent of the source, collection year, and geographic location (Figure S4.5).

As discussed earlier, the ST of the MDR CR-KV isolate G12 was not assigned by the *K. variicola* MLST scheme (Figure 4.1). From the phylogenetic analysis (Figure 4.5), it was

also evident that the G12 isolate formed a single clade distinct from the related isolates in the cluster, suggesting a new ST of *K. variicola*. The cluster containing the G12 isolate was characterised by MDR-type, ESBL- and carbapenemase-producing isolates mainly from human sources from around the world (Figure 4.5). Phylogenetic analysis also showed that wastewater isolate G12 from 2018 was related to an ST200 isolate from a pneumonia patient in the United States in 2015 (VKTF00000000.1) (Figure 4.5). However, the G12 isolate was found to harbour more resistance genes against β-lactams, including carbapenem (*bla*$_{\text{GES-5}}$ and *bla*$_{\text{LEN-2}}$), aminoglycosides (*aac(6')-Ib-cr* and *aac(6')-Ib3*), fluoroquinolones (*aac(6')-Ib-cr* and *qnrS2*), and fosfomycin (*fosA*) than related isolates from the United States, which carried only resistance genes against β-lactams (*bla*$_{\text{LEN-24}}$), fluoroquinolones (*oqxAB*), and fosfomycin (*fosA*) (Figure 4.5).

The XDR ST101 isolate G13 from wastewater found here in 2018 was very similar to MDR-type human isolates belonging to ST101 from China found in 2017 (CABWXA000000000.1) and also to ST251 from Japan found in 2014 (BIJE00000000.1) carrying identical resistance genes against β-lactams (*bla*$_{\text{LEN-7}}$), fluoroquinolones (*oqxAB*), and fosfomycin (*fosA*) (Figure 4.6). Compared to these closely related isolates, the XDR CR-KV isolate G13 possesses more resistance genes against β-lactams, including carbapenem (*bla*$_{\text{IMP-4}}$, *bla*$_{\text{OXA-48}}$, *bla*$_{\text{LEN-7}}$, and *bla*$_{\text{TEM-1B}}$), aminoglycosides (*aac(6')-Ib-cr, aac(3)-IId,* and aac(6*')-Ib3*), fluoroquinolones (*oqxAB, aac(6')-Ib-cr,* and *qnrB2*), sulfonamides (*sul1*), and fosfomycin (*fosA*) (Figure 4.6). To the best of my knowledge, the XDR ST101 clone of *K. varricola* has never been detected in Australia.

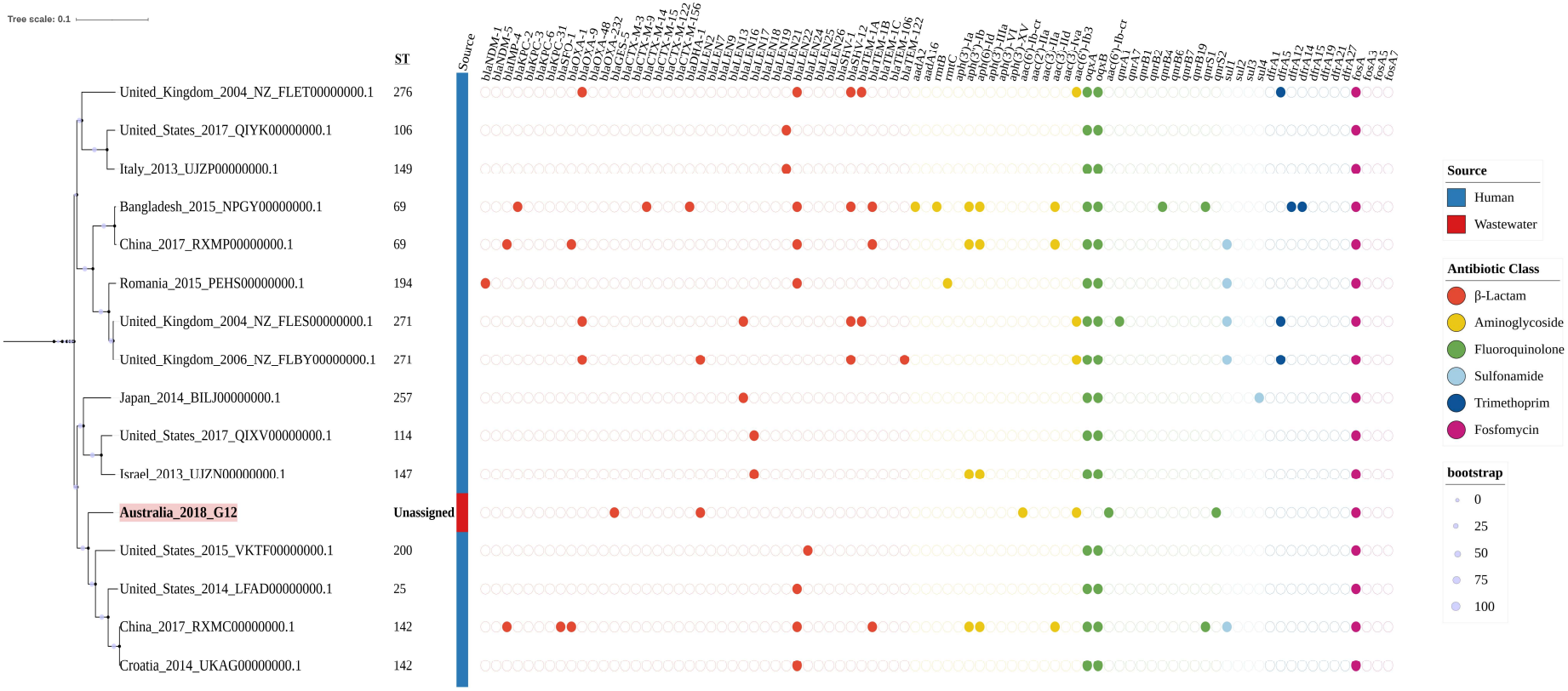


Figure 4.5 Maximum likelihood phylogeny of the *K. variicola* isolate G12 from wastewater belonging to a novel sequence type (ST) and other related global isolates (for a larger phylogenetic tree of 328 *K. variicola* isolates, see Figure S4.5). The phylogeny was inferred from the alignment of the concatenated core CDS (n = 3,336) determined by Roary and RAxML using the GTR-Gamma model and 100 bootstrap iterations. The tree nodes are labelled with the origin, year of isolation, and accession number of the isolates, followed by the STs of the isolates identified based on the *K. variicola* MLST scheme (http://mlstkv.insp.mx/). The wastewater isolate is in bold and highlighted in pink. The source of the isolates and acquired resistance genes against different clinically important antibiotic classes are annotated on the coloured strips according to the given key.

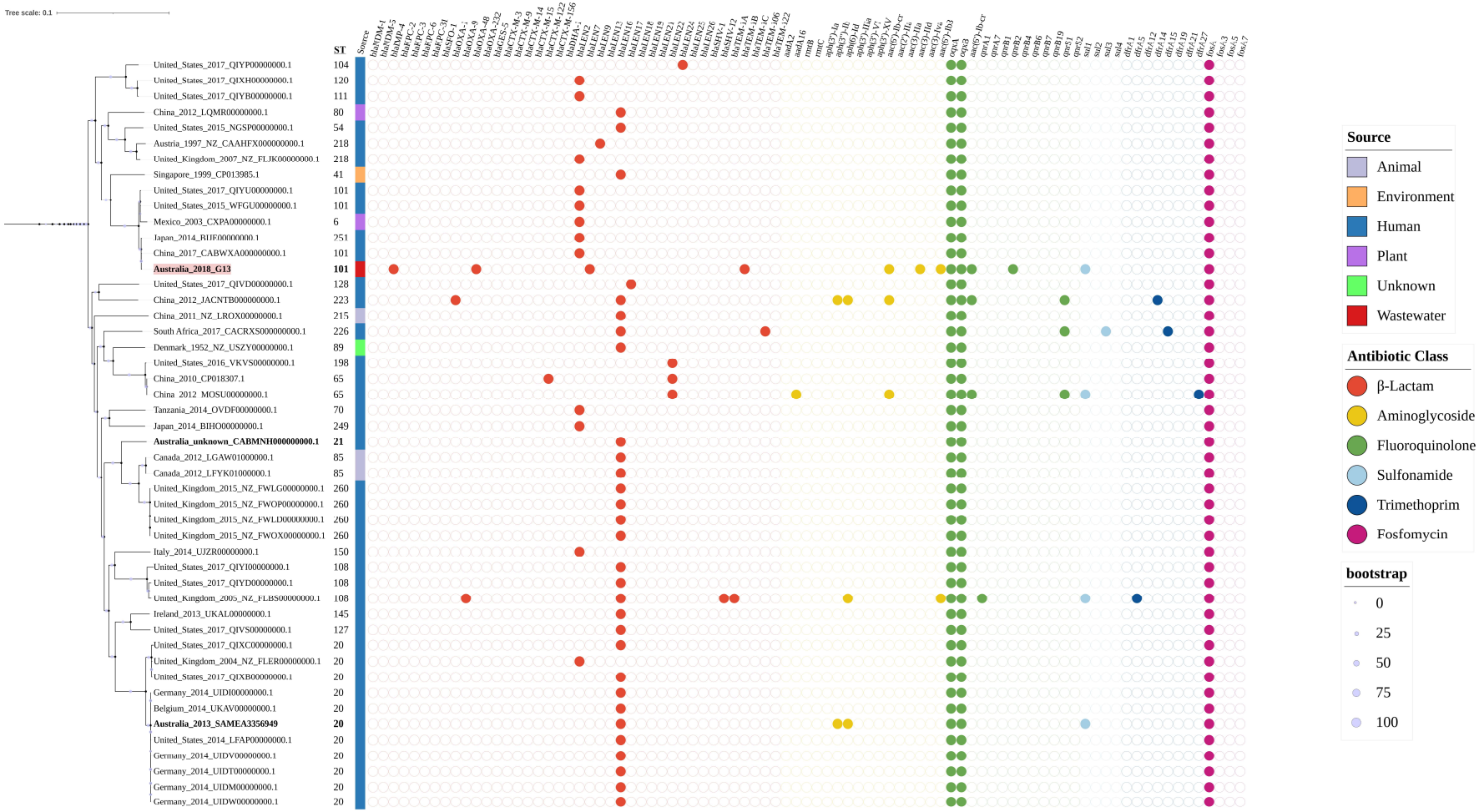


Figure 4.6 Maximum likelihood phylogeny of the *K. variicola* isolate G13 belonging to ST101 from wastewater and other related global isolates (for a larger phylogenetic tree of 328 *K. variicola* isolates, see Figure S4.5). The phylogeny was inferred from the alignment of the concatenated core CDS (n = 3,336) determined by Roary and RAxML using the GTR-Gamma model and 100 bootstrap iterations. The tree nodes are labelled with the origin, year of isolation, and accession number of the isolates, followed by the sequence types (ST) of the isolates identified based on the *K. variicola* MLST scheme (http://mlstkv.insp.mx/). The wastewater isolate is in bold and highlighted in pink. The source of the isolates and acquired resistance genes against different clinically important antibiotic classes are annotated on the coloured strips according to the given key.

Carbapenemase-producing *K. variicola* isolates have rarely been detected in Australia, and if so, mainly from human sources (AGAR, 2019; AURA, 2021). For instance, only one carbapenemase-producing *K. variicola* isolate was detected in blood samples in 2018 from Melbourne, Australia (AGAR, 2019). However, I detected two carbapenemase-producing isolates belonging to new and emerging clones of *K. variicola* from wastewater in 2018 in Sydney, Australia. This finding implies that there were somewhat reasonably abundant isolates to be detected in the Australian community. Therefore, continuous wastewater-based surveillance would be helpful to better understand the epidemiology of these relatively new *Klebsiella* strains and to track their emergence and spread within and beyond clinical settings.

Overall, phylogenetic analysis suggests that the *Klebsiella* species collected in the wastewater probably originated from human sources. Furthermore, several new clones of *K. pneumoniae* and *K. variicola* were detected for the first time in the local Australian setting by wastewater testing.

4.3.3 Antibiotic resistance determinants

The identification of several unique antibiotic resistance profiles in the XDR CR-KP and CR-KV, and MDR ESBL-KP and CR-KV isolates from wastewater led me to investigate the molecular mechanisms that confer resistance. ResFinder predicted multiple antibiotic resistance genes against clinically relevant antibiotic classes from the draft genome of wastewater isolates (Table 4.2). The detailed resistance mechanisms against each antibiotic class are discussed in the following sections.

4.3.3.1 Resistance to β-lactams is mainly attributed to the carriage of diverse genes encoding β-lactamases, ESBLs, and carbapenemases

K. pneumoniae and *K. variicola* are intrinsically resistant to ampicillin and piperacillin owing to the presence of chromosomally encoded sulfhydryl variable-1 (SHV-1)- and LEN-type β-lactamases, respectively (Haeggman et al., 2004; Potter et al., 2018). However, the most prevalent and clinically significant mechanism of β-lactam resistance in *Klebsiella* species is the acquisition of several β-lactamase genes, such as those encoding ESBLs, AmpC β-lactamases, and carbapenemases (Jacoby, 2009; Pitout et al., 2015; Navon-Venezia et al., 2017; Meini et al., 2019; Peirano & Pitout, 2019). ESBLs

primarily reported in *Klebsiella* species include CTX-M-, SHV- (i.e. a genetic variant of SHV-1), and TEM-types (Hennequin & Robin, 2016; Navon-Venezia et al., 2017; Rodríguez-Medina et al., 2019); plasmid-mediated AmpC β-lactamases include cephamycinase (CMY)-, DHA-, FOX-, and MIR-types (Jacoby, 2009; Navon-Venezia et al., 2017; Meini et al., 2019); and carbapenemases include Guiana extended-spectrum (GES)-, KPC-, IMP-, NDM-, VIM-, and OXA-48-types (Nordmann et al., 2011; Hennequin & Robin, 2016; Navon-Venezia et al., 2017; Rodríguez-Medina et al., 2019).

Carbapenem (i.e. meropenem) resistance in the XDR CR-KP isolate G14 was conferred mainly by the carriage of IMP-4-type carbapenemase (Table 4.2). In addition, this isolate carried an OXA-48-type carbapenemase that usually confers low-level resistance against meropenem (Poirel et al., 2004; Poirel et al., 2012). The IMP-4-type carbapenemase gene is also attributed to the resistance of isolate G14 to all other tested β-lactam antibiotics such as first-, second-, third-, and fourth-generation cephalosporins (i.e. cefazolin, ceftriaxone, ceftazidime, and cefepime), cephamycin (i.e. cefoxitin), and the penicillins and β-lactam inhibitors (i.e. amoxicillin/clavulanic acid, piperacillin/tazobactam, and ticarcillin/clavulanic acid) (Table 4.2). In addition to carbapenemase, isolate G14 also carried TEM-1B- and chromosomal SHV-187-type β-lactamases (Table 4.2).

Resistance to β-lactam antibiotics in ESBL-KP isolates G6, G7, and G9 was mainly attributed to the carriage of various ESBL and β-lactamase enzymes (Table 4.2). In isolates G6 and G7, resistance to cephalosporins such as cefazolin, ceftazidime, ceftriaxone, and cefepime was explained by the presence of CTX-M-type ESBL (Table 4.2). In isolate G9, resistance to cefazolin, ceftazidime, and ceftriaxone was mediated by CTX-M-type ESBL (Table 4.2). Interestingly, despite carrying resistant-conferring CTX-M-type ESBL, isolate G9 was phenotypically identified as sensitive to cefepime (Table 4.2). The promoter region of the *bla*$_{\text{CTX-M-15}}$ gene was found to be unchanged, and no deleterious mutations were found in the gene. Therefore, the mechanism for this genotype-phenotype discrepancy remains unclear. In isolates G6 and G7, amoxicillin/clavulanic acid resistance was attributed to the carriage of OXA-1-type β-lactamases (Table 4.2). Interestingly, regardless of the presence of OXA-1-type β-lactamases, isolate G9 was sensitive to amoxicillin/clavulanic acid (Table 4.2). As no alterations in the promoter region and the *bla*$_{\text{OXA-1}}$ gene were found, the mechanism of

discordance between amoxicillin/clavulanic acid resistance genes and the susceptibility of isolate G9 remains unclear. Although ESBL-KP isolates G6 and G7 were phenotypically detected as ticarcillin/clavulanic acid resistant, no resistance determinants such as CMY-type AmpC β-lactamases were observed (Jacoby, 2009), indicating unknown mechanism of resistance. In addition to the ESBL and β-lactamase genes mentioned above, isolates G6, G7, and G9 had chromosomal SHV-type β-lactamase, and isolates G6 and G9 carried TEM-1B-type β-lactamase (Table 4.2).

In XDR CR-KV isolate G13, carbapenem resistance was mediated mainly by IMP-4-type carbapenemase (Table 4.2). This isolate also carried low-level meropenem resistance-conferring OXA-48-type carbapenemase (Poirel et al., 2004; Poirel et al., 2012). The resistance of the G13 isolate to all other tested β-lactam antibiotics such as cephalosporins, cephamycin, and penicillins and β-lactam inhibitors can also be explained by the presence of IMP-4-type carbapenemase (Table 4.2). In addition to carbapenemase, this isolate carried a TEM-1B-type β-lactamase and a chromosomal LEN-7-type β-lactamase (Table 4.2). In the MDR CR-KV isolate G12, resistance to β-lactam antibiotics could be explained by the carriage of a GES-5-type carbapenemase (Table 4.2), which provides intermediate susceptibility to meropenem (Table 4.1) (Bae et al., 2007; Walther-Rasmussen & Høiby, 2007; Frase et al., 2009) and resistance to cephalosporins, cephamycin, and penicillins and β-lactam inhibitor such as amoxicillin/clavulanic acid (Table 4.2). Although isolate G12 exhibited resistance to piperacillin/tazobactam and ticarcillin/clavulanic acid, no previously reported resistance determinants such as KPC-, MBL-, and OXA-48-type carbapenemases (Nordmann et al., 2011; Navon-Venezia et al., 2017; Rodríguez-Medina et al., 2019) or CMY-type AmpC β-lactamases were found (Jacoby, 2009), indicating unknown resistance mechanisms. In addition to carbapenemase, isolate G12 had a chromosomal LEN-2-type β-lactamase (Table 4.2).

Table 4.2 Genetic characteristics of the carbapenem-resistant (CR) and extended-spectrum β-lactamases (ESBL)-producing *K. pneumoniae* (KP) and *K. variicola* (KV) isolates from wastewater.

Isolates	MLST types	Phenotypes	Antibiotic resistance pattern[#]	Antibiotic resistance genes	Virulence-associated genes	Plasmid replicons
G14	2791	CR-KP	AMP, FOX, CFZ, CAZ, CRO, FEP, MEM, AMC, TZP, TIM, GEN, CIP, NOR, TMP, SXT, NIT	*bla*$_{IMP-4}$, *bla*$_{OXA-48}$, *bla*$_{TEM-1B}$, *bla*$_{SHV-187}$, *aac(3)-IId, aac(6')-Ib-cr*, *aac(6')-Ib3*, *oqxA, oqxB, qnrA1, qnrB2, sul1, nfsA* (T211I)	*entABCDEF, fimACDFGH, mrkABCDFHIJ, kfuABC*	Col (MGD2), Col (pHAD28), Col440I, Col440II, FII (pBK30683), IncFIB (K), IncFII (K), IncFII (pKP91), IncM2, IncR
G6	13	ESBL-KP	AMP, CFZ, CAZ, CRO, FEP, AMC, TIM, GEN, TOB, CIP, TMP, SXT, NIT	*bla*$_{OXA-1}$, *bla*$_{CTX-M-15}$, *bla*$_{TEM-1B}$, *bla*$_{SHV-101}$, *aac(3)-IIa, aac(6')-Ib-cr*, *parC* (N304S),*oqxA, oqxB, qnrB1*, *dfrA14*, *sul2*	*entABCDEF, fimACDFGH, mrkABCDFHIJ, kfuABC, fyuA, irp1, irp2, ybtEPQSTUX, clbABCDEFGHIJLMNOPQR*	Col440II, ColRNAI, IncFIB (K), IncFII (K)
G7	353	ESBL-KP	AMP, CFZ, CAZ, CRO, FEP, AMC, TIM, GEN, TOB, CIP, TMP, SXT, NIT	*bla*$_{OXA-1}$, *bla*$_{CTX-M-15}$, *bla*$_{SHV-187}$, *aac(3)-IId, oqxA, oqxB, qnrB2, dfrA17, sul2*	*entABCDEF, fimACDFGH, mrkABCDFHIJ, fyuA, irp1, irp2, ybtAEPQSTUX*	Col (MGD2), Col (pHAD28), Col440I, Col440II, FII (pBK30683), IncFIB(K), IncFII (K), IncM2, IncR
G9	268	ESBL-KP	AMP, CFZ, CAZ, CRO, TMP, SXT, NIT	*bla*$_{OXA-1}$, *bla*$_{CTX-M-15}$, *bla*$_{SHV-11}$, *bla*$_{TEM-1B}$, *aac(6')-Ib-cr*, *oqxA, oqxB, dfrA12*, *sul1*	*entABCDEF, fimACDFGH, mrkABCDFHIJ, fyuA, irp1, irp2, ybtAEPQSTUX*	Col (pHAD28), Col156, Col440I, IncFIB (AP001918), IncFIB (K), IncFII (29), IncFII (K)

G13	101	CR-KV	AMP, FOX, CFZ, CAZ, CRO, FEP, MEM, AMC, TZP, TIM, GEN, CIP, TMP, SXT, NIT	*bla*$_{IMP-4}$, *bla*$_{OXA-48}$, *bla*$_{LEN-7}$, *bla*$_{TEM-1B}$, *aac(3)-IId, aac(6')-Ib-cr*, *aac(6')-Ib3*, *oqxA, oqxB, qnrB2, sul1*	*entABCDEF, fimACDFGH, mrkABCDFHIJ, kfuABC*	Col (pHAD28), IncFIA (HI1), IncFIB (K), IncFII (K), IncM2
G12	Unknown	CR-KV	AMP, FOX, CFZ, CAZ, CRO, FEP, AMC, TZP, TIM	*bla*$_{GES-5}$, *bla*$_{LEN-2}$, *aac(6')-Ib-cr*, *aac(6')-Ib3*, *qnrS2*	*entABCDEF, fimACDFGH, mrkABCDFHIJ, kfuABC*	IncQ2

[#]Here, **AMP** = Ampicillin, **FOX** = Cefoxitin, **CFZ** = Cefazolin, **CAZ** = Ceftazidime, **CRO** = Ceftriaxone, FEP = Cefepime, **MEM** = Meropenem, **AMC** = Amoxicillin/clavulanic acid, **TZP** = Piperacillin/tazobactam, **TIM**= Ticarcillin/clavulanic acid, **GEN**= Gentamicin, **TOB** =Tobramycin, **CIP** = Ciprofloxacin, **NOR** = Norfloxacin, **TMP** = Trimethoprim, **SXT** = Trimethoprim-Sulfamethoxazole, **NIT** = Nitrofurantoin.

4.3.3.2 Resistance to aminoglycosides is mainly associated with genes that encode various aminoglycoside acetyltransferases

The most prevalent determinant of aminoglycoside resistance in *Klebsiella* species is the production of different aminoglycoside-modifying enzymes, of which three classes are defined based on their modifying activities such as acetyltransferases (AAC), nucleotidyltransferases (ANT) and phosphotransferases (APH) (Shaw et al., 1993; Ramirez & Tolmasky, 2010; Ramirez et al., 2013). Another clinically important aminoglycoside resistance mechanism is the acquisition of plasmid-encoded 16S ribosomal RNA (rRNA) methyltransferases (16S RMTases) such as *armA, rmtB-H,* and *npmA* that confer high-level resistance to nearly all clinically available aminoglycosides (Galimand et al., 2003; Doi et al., 2016; Wachino et al., 2020).

Resistance to aminoglycosides in this study's isolates was mainly mediated by various aminoglycoside acetyltransferases encoding genes (Table 4.2). Resistance to gentamicin in the CR-KV isolate G14 can be explained by the presence of an *aac(3)-IId* gene encoding for the aminoglycoside 3-N-acetyltransferase type IId (Table 4.2), as previously reported (Shaw, 1993; Ramirez & Tolmasky, 2010). Although isolate G14 carried tobramycin resistance-conferring genes such as *aac(3)-IId*, *aac(6')-Ib-cr* and *aac(6')-Ib3*, and amikacin resistance genes such as *aac(6')-Ib-cr* and *aac(6')-Ib3* (Shaw, 1993; Ramirez & Tolmasky, 2010; Ramirez et al., 2013), it was phenotypically detected to be sensitive to these antibiotics (Table 4.1). As no deleterious mutations were found in these genes and the promoter regions were found to be unaltered, the mechanisms for these genotype-phenotype discrepancies remain unclear. In ESBL-KP isolate G7, the presence of the *aac(3)-IId* gene was correlated with resistance to gentamicin and tobramycin (Table 4.2). Resistance to gentamicin and tobramycin in the ESBL-KP isolate G6 was attributed to the carriage of the *aac(3)-IIa* gene encoding aminoglycoside 3-N-acetyltransferase type IIa (Table 4.2), as per previous studies (Shaw, 1993; Ramirez & Tolmasky, 2010).

In CR-KV isolate G13, resistance to gentamicin was attributed to the carriage of the *aac(3)-IId* gene (Table 4.2). Despite carrying the *aac(6')-Ib-cr* and *aac(6')-Ib3* genes, the CR-KV isolates G13 and G12 were phenotypically identified as intermediately susceptible to tobramycin and susceptible to amikacin (Table 4.1, Table 4.2). The promoter regions of the *aac(6')-Ib-cr* and *aac(6')-Ib3* genes were found to be unchanged,

and no deleterious mutations were found in these genes. Therefore, the mechanism of discordance between the tobramycin and amikacin resistance genes and the susceptibility of isolates G13 and G12 remains unclear.

4.3.3.3 Fluoroquinolone resistance is mainly caused by the carriage of multiple PMQR determinants and the likely overexpressed MDR efflux pump

The clinically relevant level of fluoroquinolone (i.e. ciprofloxacin and norfloxacin) resistance in *Klebsiella* species is mediated primarily by mutations in the quinolone resistance determining region (QRDR) of the antibiotic's target enzymes, such as DNA gyrase (*gyrA-gyrB* subunit) and topoisomerase IV (*parC-parE* subunit) (Chen et al., 2003; Ruiz, 2003; Jacoby, 2005; Hooper & Jacoby, 2016). In addition, acquisition of plasmid-mediated quinolone resistance (PMQR) determinants, such as the *aac(6')-Ib-cr* gene encoded for an aminoglycoside acetyltransferase that can modify certain fluoroquinolones*; qnr*-type genes encoding pentapeptide repeat proteins that physically protect DNA gyrase and topoisomerase IV from inhibition by fluoroquinolones; *oqxAB*- and *qepA*-type genes encoded for efflux pumps could confer a low-level of fluoroquinolone resistance (Martínez-Martínez et al., 1998; Rodríguez-Martínez et al., 2003; Robicsek et al., 2006; Jacoby et al., 2014; Wong et al., 2015). However, a high level of fluoroquinolone resistance can be mediated by the overexpression of efflux pumps, such as those encoded by the *oqxAB* and *acrAB* genes (Mazzariol et al., 2002; Padilla et al., 2010; Bialek-Davenet et al., 2015; Wong et al., 2015).

Resistance to ciprofloxacin and norfloxacin in this study's isolates could be mainly attributed to the acquisition of PMQR determinants (Table 4.2), as these isolates did not have any deleterious or previously reported resistance-associated mutations in the GyrAB and ParCE proteins (Deguchi et al., 1997; Chen et al., 2003; Ruiz, 2003; Hooper & Jacoby, 2016; Mirzaii et al., 2018). For example, reduced susceptibility to ciprofloxacin in CR-KP isolate G14 was attributed to the presence of multiple PMQR determinants such as the *aac(6')-Ib-cr*, *oqxAB*, *qnrA1,* and *qnrB2* genes (Table 4.2) that were reported to act additively to confer resistance (Robicsek et al., 2006; Schultsz & Geerlings, 2012). The isolate G14 also had a noble insertion of 19 amino acids (Ins MVHTFTNVCKRNLCKVINL (1-19)) in the conserved N-terminal region of the AcrAB efflux pump repressor, such as *acrR* (Schneiders et al., 2003; Padilla et al., 2010).

However, no deleterious mutations were found in other AcrAB regulators such as *ramA*, *ramR,* and *soxS* (Schneiders et al., 2003; Hentschke et al., 2010; Bialek-Davenet et al., 2011; Xu et al., 2021). Therefore, the large insertion in *acrR* could have an adverse effect on AcrAB repression and thus may increase the expression of this efflux pump in isolate G14 (Wang et al., 2001; Schneiders et al., 2003). In the ESBL-KP isolate G6, resistance to ciprofloxacin can be explained by the carriage of multiple PMQR determinants such as the *oqxAB*, *aac(6')-Ib-cr,* and *qnrB1* genes and a deleterious mutation in ParE (N304S) (Table 4.2). Ciprofloxacin resistance in the ESBL-KP isolate G7 was probably caused by the co-carriage of the *oqxAB* and *qnrB2* genes (Table 4.2). In the CR-KV isolate G13, resistance to ciprofloxacin can be attributed to the presence of the *oqxAB*, *aac(6')-Ib-cr,* and *qnrB2* genes (Table 4.2). Interestingly, despite carrying PMQR determinants such as the *aac(6')-Ib-cr* and *qnrS2* genes (Table 4.2), the CR-KV isolate G12 was sensitive to ciprofloxacin and norfloxacin (Table 4.1). No deleterious mutations were found in the promoter region of these genes. Therefore, the mechanism of this genotype-phenotype discrepancy remains unclear.

4.3.3.4 Resistance to trimethoprim and sulfamethoxazole is mainly attributed to the carriage of genes that encode drug-resistant variants of chromosomal target enzymes

In *Klebsiella* species, resistance to sulfonamide (i.e. sulfamethoxazole) and trimethoprim is mainly attributed to the acquisition of *sul*- and *dfr*-type genes, respectively (Huovinen et al., 1995; Huovinen, 2001; Sköld, 2001). The *sul*-type genes encode for an antibiotic-resistant variant of the chromosomal target enzyme dihydropteroate synthase that catalyses the first step of folate biosynthesis required for the *de novo* synthesis of nucleotides and amino acid acids (i.e. glycine, histidine, methionine, and serine) (Huovinen et al., 1995; Sköld, 2001; Naderi & House, 2018). The *dfr*-type genes encode for a drug-resistant variant of the target enzyme dihydrofolate reductase, which catalyses the final step of folate biosynthesis (Huovinen et al., 1995; Huovinen, 2001; Sköld, 2001). In addition, resistance to trimethoprim can also be mediated by the OqxAB efflux pump in *Klebsiella* species (Hansen et al., 2007; Li et al., 2019).

Trimethoprim resistance in the CR-KP isolate G14 can be explained by the carriage of the *oqxA* and *oqxB* genes encoding the OqxAB efflux pump, and the resistance to

trimethoprim-sulfamethoxazole was mediated by the co-occurrence of the *oqxAB* and *sul1* genes (Table 4.2), as previously documented (Huovinen, 2001; Sköld, 2001; Frank et al., 2007; Hansen et al., 2007; Li et al., 2019). In ESBL-KP isolates G6, G7, and G9, resistance to trimethoprim was mainly attributed to the carriage of *dfrA*-type genes and resistance to trimethoprim-sulfamethoxazole was conferred by the co-carriage of *dfrA*- and *sul*-type genes (Table 4.2), as per previous studies (Huovinen, 2001; Sköld, 2001; Frank et al., 2007; Brolund et al., 2010). In addition, isolates G6, G7, and G9 also carried the *oqxAB* genes (Table 4.2). In the CR-KV isolate G13, resistance to trimethoprim was attributed to the presence of the *oqxAB* genes, and the trimethoprim-sulfamethoxazole resistance can be explained by the co-occurrence of the *oqxAB* and *sul1* genes (Table 4.2).

4.3.3.5 Resistance to nitrofurantoin is mediated primarily by MDR efflux pumps

Resistance to nitrofurantoin in the *Klebsiella* species is likely mediated by loss-of-function mutations in the chromosomal *nfsA* and/or *nfsB* genes encoding oxygen-insensitive nitroreductases that catalyse the reduction of nitrofurantoin into toxic intermediate compounds (Sandegren et al., 2008). In addition, inactivation of the *ribE* gene encoding 6,7-dimethyl-8-ribityllumazine synthase involved in the reduction of nitrofurantoin has been reported to confer resistance to nitrofurantoin (Vervoort et al., 2014). Furthermore, multidrug efflux pumps OqxAB and/or AcrAB have also been reported to be associated with resistance to nitrofurantoin (Ho et al., 2015; Li et al., 2019; Xu et al., 2019).

Comparison of nitrofurantoin-resistant CR-KP isolate G14, and ESBL-KP isolates G6, G7 and G9 with the nitrofurantoin-sensitive *K. pneumoniae* strain NICU_2_P7 (GenBank accession number, NZ_CP060049.1) revealed that none of these isolates had deleterious mutations in the *nfsB* and *ribE* gene. A novel deleterious mutation in the *nfsA* (T211I) gene was found only in isolate G14. This mutation (T211I), together with the carriage of the efflux pump encoding genes *oqxAB* (Table 4.2) and the likely overexpressed AcrAB efflux pump due to a large insertion in the regulatory *acrR* gene (as described above), could be associated with nitrofurantoin resistance in isolate G14, as described in previous studies (Ho et al., 2015; Osei Sekyere, 2018; Li et al., 2019; Xu et al., 2019). Resistance to nitrofurantoin in isolates G6, G7, and G9 is likely mediated by the *oqxAB* genes (Table 4.2), as previously reported (Ho et al., 2015; Osei Sekyere, 2018; Li et al., 2019).

Similarly, nitrofurantoin resistance in the CR-KV isolate G13 could be attributed to the carriage of the *oqxAB* genes (Table 4.2), as no deleterious mutation was found in the *nfsAB* and *ribE* genes compared to the nitrofurantoin-sensitive *K. variicola* strain 179 (BioSample accession number, SAMN0445659).

In general, the resistance of *Klebsiella* species to β-lactams, aminoglycosides, fluoroquinolones, trimethoprim and sulfamethoxazole, and nitrofurantoin was primarily mediated by the carriage of various previously defined clinically significant resistance-conferring determinants. In addition, unknown mechanisms of resistance against some antibiotics such as piperacillin/tazobactam and ticarcillin/clavulanic acid were observed.

4.3.4 Virulence gene content indicates infections or asymptomatic carriage

Klebsiella species usually harbour core chromosomally encoded virulence factors required for opportunistic infections in vulnerable individuals, including capsular polysaccharide (K antigen), lipopolysaccharide (O antigen), siderophore enterobactin (Ent), and adhesins (i.e. type I and type III fimbriae) (Holt et al., 2015; Paczosa et al., 2016; Rodríguez-Medina et al., 2019; Wyres et al., 2020). However, several chromosomal and acquired virulence factors are reported to cause increased virulence or 'hypervirulence' and are associated with severe community-acquired infections. These include serum-resistant capsular serotypes such as K1, K2, K5, K20 and K57 in *K. pneumoniae* (Chung et al., 2007; Fang et al., 2007; Yu et al., 2008; Shon et al., 2013; Hirai et al., 2016; Choby et al., 2020), and K16 in *K. variicola* (Lu et al., 2018); capsule overproduction or hypermucoidy due to the acquisition of regulator genes *rmpA* or *rmpA2* (Lai et al., 2003; Cheng et al., 2010)*;* acquired siderophores such as aerobactin (Iuc), salmochelin (Iro), and yersiniabactin (Ybt) (Nassif & Sansonetti, 1998; Carniel, 2001; Müller et al., 2009; Holt et al., 2015; Paczosa et al., 2016); and the genotoxin colibactin (Clb) (Lu et al., 2017). The genetic relatedness of *Klebsiella* isolates from wastewater with strains from human sources led me to investigate whether these MDR and XDR wastewater isolates were also associated with infections in humans. For this purpose, I used the Institut Pasteur's BIGSdb that predicted multiple virulence factors encoding genes from the draft genome of the study isolates (Table 4.2).

The XDR CR-KP isolate G14 harboured several chromosomally encoded virulence factors, including the capsular serotype K48, the lipopolysaccharide (LPS) serotype O1 (subtype O2v1), the siderophore enterobactin encoded by the *entABCDEF* gene cluster, the type I fimbriae encoding *fimACDFGH* gene cluster, and the type III fimbriae encoded by the *mrkABCDFHIJ* gene cluster (Table 4.2). In addition, isolate G14 carried a ferric iron uptake system encoding *kfuABC* operon (Table 4.2) that was reported to be associated with hypervirulence in *K. pneumoniae* (Ma et al., 2005). However, based on the presence of mainly core chromosomally encoded virulence factors and the lack of other hypervirulence-associated molecular markers such as *rmpA* or *rmpA2, iuc* and *iro* (Russo et al., 2018), it can be inferred that isolate G14 was associated with either opportunistic infections in vulnerable individuals or asymptomatic carriage in healthy individuals (Podschun & Ullmann, 1998; Holt et al., 2015).

The MDR ESBL-KP isolate G6 harboured multiple chromosomally encoded virulence factors such as capsular serotype K3, LPS serotype O1v2, enterobactin (*entABCDEF*), type I fimbriae (*fimACDFGH*), and type III fimbriae (*mrkABCDFHIJ*) (Table 4.2). In addition, isolate G6 had the genotoxin colibactin encoded by the *clbABCDEFGHIJLMNOPQR* gene cluster, the ferric iron uptake operon *kfuABC*, and the siderophore yersiniabactin-related genes, such as *irp1* and *irp2* encoding yersiniabactin biosynthesis proteins, the *ybtEPQSTUX* gene cluster encodes for yersiniabactin biosynthesis and transport proteins and *fyuA* encoding the yersiniabactin receptor (Table 4.2) (Carniel, 2001; Paczosa et al., 2016). The *ybt* locus encoding yersiniabactin was found to be a strong predictor of infection versus intestinal carriage in humans (Holt et al., 2015) and the genotoxin colibactin (*clb*) that induces DNA damage in eukaryotes was also found to be associated with serious infections, such as liver abscess and the development of meningitis (Lai et al., 2014; Lu et al., 2017). Therefore, the presence of yersiniabactin, colibactin, and the ferric iron uptake system in isolate G6 is indicative of its potential association with human infections. However, due to the apparent lack of other molecular markers associated with hypervirulence, isolate G6 cannot be classified as hypervirulent (Russo et al., 2018). ESBL-KP isolates G7 and G9 also had multiple chromosomally encoded virulence factors such as enterobactin (*entABCDEF*), type I fimbriae (*fimACDFGH*), and type III fimbriae (*mrkABCDFHIJ*) (Table 4.2). In addition, isolate G7 had unknown capsular and OL104-type LPS, isolate G9 had hypervirulence-

associated K20-type capsule and O2v1-type LPS, and both isolates carried yersiniabactin-related genes (Table 4.2). The presence of yersiniabactin in isolates G7 and G9 indicates that these isolates were associated with human infections, as per previous studies (Lawor et al., 2007; Bachman et al., 2011; Holt et al., 2015). Despite the fact that isolate G9 had a hypervirulence-associated capsule (i.e. K20), it could not be classified as hypervirulent due to the absence of other genetic markers (Russo et al., 2018).

The XDR CR-KV isolate G13 and MDR CR-KV isolate G12 mainly had several chromosomally encoded virulence factors such as enterobactin (*entABCDEF*), type I fimbriae (*fimACDFGH*), and type III fimbriae (*mrkABCDFHIJ*) (Table 4.2). In addition, isolate G13 had unknown capsular and LPS serotypes, isolate G12 had an unknown capsular type and O3-type LPS (subtype O3a), and both isolates carried the hypervirulence-associated ferric iron uptake operon *kfuABC* (Table 4.2). Since CR-KV isolates G12 and G13 lacked other hypervirulence-associated markers and had mainly chromosomally encoded virulence factors, it can be inferred that these isolates were associated with opportunistic infections or asymptomatic carriage (Podschun and Ullmann, 1998; Holt et al., 2015).

In general, the virulence gene content of the MDR ESBL-KP isolates G6, G7 and G9 indicated that they were likely associated with human infections other than carriage. However, the presence of mainly chromosomally encoded virulence factors in CR-KP isolate G14 and CR-KV isolates G12 and G13 from wastewater suggested their possible association with opportunistic infections in vulnerable individuals or asymptomatic carriage in healthy people.

4.3.5 The potential for the acquisition and transfer of antibiotic resistance genes

Klebsiella species have been considered one of the important sources and shuttles for antibiotic resistance throughout the world (Ramirez et al., 2014; Navon-Venezia et al., 2017). Mobile genetic elements such as plasmids are considered the main means of acquiring and disseminating antibiotic resistance genes within and between bacterial species (Carattoli, 2013; Rozwandowicz et al., 2018). The identification of various resistance genes in *Klebsiella* isolates from wastewater led me to investigate the plasmid

content and associated resistance genes to determine whether these isolates have acquired or could transfer these genes. PlasmidFinder predicted multiple replicons indicative of respective plasmids from the draft genome of XDR and MDR *Klebsiella* isolates from wastewater (Table 4.2). Manual inspection of contigs with plasmid replicons suggested plasmid-mediated acquisition of resistance genes in some *Klebsiella* isolates.

The XDR CR-KP isolate G14 harboured various colicinogenic (Col)-, IncF (multi-replicon)-, IncM- and IncR-type plasmid replicons (Table 4.2). In this isolate, the contig harbouring IncM2 plasmid replicon also had the β-lactamase encoding gene $bla_{\text{TEM-1B}}$ and the aminoglycoside acetyltransferase encoding gene *aac(3)-IId*, suggesting plasmid-mediated acquisition of these genes (Table S4.4). This finding is consistent with previous studies that reported the dissemination of the $bla_{\text{TEM-1B}}$ and *aac(3)-IId* genes by the IncM2 plasmid (Dor et al., 2020; Palmieri et al., 2021). The MDR ESBL-KP isolates G6, G7, and G9 had several Col- and IncF-type plasmid replicons in various combinations (Table 4.2). The isolate G7 also had IncM- and IncR-type plasmid replicons (Table 4.2). The XDR CR-KV isolate G13 had several Col-, IncF- and IncM-type plasmid replicons (Table 4.2). The MDR CR-KV isolate G12 had an IncQ-type plasmid replicon that may be associated with the carriage of the PMQR gene *qnrS2* (Table S4.4), as previously documented (Han et al., 2012; Wen et al., 2016). I thus found evidence for the acquisition of several clinically important antibiotic resistance genes through plasmids in XDR CR-KP and MDR CR-KV isolates from wastewater.

4.4 Conclusion

In this study, I found that the XDR CR-KP and CR-KV, and MDR ESBL-KP and CR-KV isolates from wastewater were resistant to multiple critically important antibiotics. In addition, I identified that these isolates were phylogenetically related to globally disseminated and emerging human-associated MDR-type clones, some of which were detected for the first time in local Australian settings. Furthermore, I identified that resistance against most of the critically important antibiotics was conferred by various known mechanisms and found indications for novel resistance mechanisms against piperacillin/tazobactam and ticarcillin/clavulanic acid. Moreover, I found that MDR ESBL-KP isolates were likely associated with human infections other than carriage due to the presence of multiple virulence determinants related to infection. In contrast, XDR

CR-KP, CR-KV and MDR CR-KV isolates carrying only chromosomally encoded virulence factors could be associated with opportunistic infections in vulnerable individuals or asymptomatic carriage in healthy people. I also found evidence that XDR CR-KP and MDR CR-KV isolates associated with opportunistic infections or asymptomatic carriage could serve as important reservoirs of transferrable resistance genes. Overall, I found that wastewater-based surveillance has the clear potential to monitor antibiotic resistance in isolates associated with infections and asymptomatic carriage and, therefore, can complement healthcare infection surveillance to guide infection control and prevention strategies to effectively track and control the emergence and dissemination of resistance in the wider community.

Chapter 5 General Discussion

This thesis has investigated the potential of wastewater-based surveillance to monitor the distribution and trends of antibiotic-resistant bacteria in the community. Through repeated testing of untreated wastewater, I was able to monitor the geospatial-temporal trends of four antibiotic-resistant pathogens in the urban area of Greater Sydney, Australia, and identify factors driving their distribution in the community. The findings of the study showed that wastewater-based surveillance of antibiotic-resistant pathogens can be done routinely and is not particularly time-consuming. In addition, I was able to identify novel bacterial isolates with unusual resistant patterns using this method. Furthermore, the genomic characterisation of selected wastewater isolates allowed me to identify their relatedness with known lineages, the genetic bases of their resistance to critically important antibiotics, their pathogenic potential, and their potential for acquisition and spread of resistance genes.

5.1 The potential of wastewater-based surveillance to monitor antibiotic resistance trends in the community

Chapter 2 has shown that wastewater-based surveillance can be used as a reliable and affordable mass-population monitoring method for geospatial-temporal and demographic trends of antibiotic resistance in clinically important bacteria in the community. Repeated testing of untreated wastewater from 25 wastewater treatment plants (WWTPs) sampled between 2017 and 2019 consistently detected extended-spectrum β-lactamases-producing *Enterobacteriaceae* (ESBL-E) isolates at the endemic level in the Sydney community. In addition, strong geospatial-temporal dynamics were observed for carbapenem-resistant *Enterobacteriaceae* (CRE), vancomycin-resistant enterococci (VRE), and methicillin-resistant *Staphylococcus aureus* (MRSA) isolates. This study provides a comprehensive and better understanding of the geospatial-temporal development of antibiotic resistance in the community than previous studies in Australia and overseas monitored antimicrobial resistance (AMR) trends in pathogens either in a single WWTP or at a single time point (Thompson et al., 2013; Kwak et al., 2015; Meir-Gruber et al., 2016; Hendriksen et al., 2019; Blaak et al., 2021). Although hospital or healthcare infection surveillance is necessary to capture AMR for individual patients, my wastewater-based monitoring offers a reliable, cost-effective, and non-intrusive method to assess changes in the

distribution and trends in AMR at the level of the non-hospitalised community. Such information could be used for early alerts of an unexpected rise in resistance in the community, for example, during outbreaks or epidemics.

The coupling between georeferenced WWTP catchment maps and publicly available demographic, socioeconomic, and healthcare infection-related information (as described by Tscharke et al., 2019) allowed me to correlate these data with the antibiotic-resistant bacterial load within each wastewater catchment to identify the factors driving their distribution in the community (Chapter 2). The results have shown that the flow normalised relative (FNR) load of resistant bacteria in wastewater was not strongly influenced by any healthcare infection-related factors, except for the CRE load, that was correlated with the average length of hospital stay (adjusted $R^2 = 0.40$, $p = 0.005$). For instance, the ESBL-E load in wastewater was driven by small contributions (adjusted $R^2 = 0.36$, $p < 0.001$) of demographic and healthcare infection-related variables such as the population between 19 to 50 years of age, completion of vocational education, and the average length of hospital stay. Surprisingly, the VRE load was mainly influenced by the number of schools (adjusted $R^2 = 0.42$, $p < 0.001$) and not by parameters related to hospitals or healthcare where infections primarily in vulnerable individuals, such as elderly and immunocompromised patients, occur (CDC, 2013; Sengupta et al., 2013; AURA, 2019). Identifying the factors driving the distribution of antibiotic-resistant bacteria beyond clinical settings could help develop effective interventions to manage and mitigate their emergence and dissemination in the community.

In recent years, the pandemic of coronavirus disease 2019 (COVID-19) has sparked an explosion of interest in wastewater-based surveillance, also known as wastewater-based epidemiology (WBE). This is evident from several studies around the world that have used WBE successfully and extensively to monitor severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) ribonucleic acid (RNA) in wastewater as an early warning system for the prevalence of symptomatic and asymptomatic COVID-19 infections in the populations of WWTP catchments (Ahmed et al., 2020; Gonzalez et al., 2020; La Rosa et al., 2020; Medema et al., 2020; Peccia et al., 2020; Thompson et al., 2020; Wu et al., 2020; D'Aoust et al., 2021; Izquierdo-Lara et al., 2021; Saththasivam et al., 2021; Barua et al., 2022; Monteiro et al., 2022; Shah et al., 2022). Such information was used, along

with clinical data, to identify outbreak hotspots or geographic locations of infection clusters, monitor infection trends in the community, and inform decision-makers about reopening efforts and directing resources, such as tests and vaccines, toward a specific area (Lodder & de Roda Husman, 2020; Medema et al., 2020; Venugopal et al., 2020; Ahmed et al., 2021; Saththasivam et al., 2021). These successful wastewater-based COVID-19 monitoring studies further support the idea that WBE can be used similarly to detect and manage the emergence and dissemination of antibiotic resistance in clinically important pathogens in the community.

5.2 Genomic features and origin of wastewater isolates

In Chapters 3 and 4, genome sequencing revealed the nature and origin of selected ESBL-E and CRE wastewater isolates resistant to most of the critically important antibiotics for human health. Genomic sequencing and phylogenetic analysis showed the relatedness of wastewater *E. coli* (EC) isolates with high-risk human-associated pandemic clones (ST131, ST167, and ST410) and emerging human-associated clones (ST1702) (Chapter 3), and *K. pneumoniae* (KP) isolates with globally disseminated and emerging human-associated clones (ST13 and ST268) (Chapter 4). Some of the interesting findings include the relatedness of an EC isolate with non-human-associated clones (ST9586), detection of a new human-associated *K. variicola* (KV) clone, and identification of some human-associated KP (ST353 and ST2791) and KV clones (ST101) for the first time in Australia.

Genomic analysis also revealed various known resistance mechanisms against critically important antibiotics such as β-lactams, aminoglycosides, fluoroquinolones, and trimethoprim and sulfamethoxazole for EC and *Klebsiella* isolates (Chapters 3 and 4). Interestingly, novel resistance mechanisms against nitrofurantoin were found for some EC isolates (Chapter 3), indicated by novel deleterious mutations in nitroreductase encoding genes (*nfsAB*) and lack of known resistance mechanisms (Sandegren et al., 2008; Vervoort et al., 2014; Ho et al., 2015; Zhang et al., 2018). In addition, the genomic analysis revealed novel mechanisms underlying resistance to piperacillin/tazobactam and ticarcillin/clavulanic acid for some *Klebsiella* isolates (Chapter 4). This is evident from the absence of previously reported resistance determinants such as KPC-, MBL- and OXA-48-type carbapenemases (Nordmann et al., 2011; Navon-Venezia et al., 2017;

Rodríguez-Medina et al., 2019) or CMY-type AmpC β-lactamases (Jacoby, 2009) in these *Klebsiella* isolates.

Analysis of virulence gene content indicates that all the ESBL-producing EC isolates were associated with extraintestinal infections in humans and animals (Chapter 3), and ESBL-producing *Klebsiella* isolates were related to human infections (Chapter 4). An interesting observation is evidence for a likely asymptomatic carriage for all the human-associated carbapenem-resistant (CR) EC and *Klebsiella* isolates based on the virulence gene content. For example, despite carrying various genes related to extraintestinal and intestinal pathotypes, CR-EC isolates were not classified into known pathotypes due to the lack of the required number of virulence markers (Chapter 3) (Kimata et al., 2005; Johnson et al., 2008; Spurbeck et al., 2012; Fujioka et al., 2013). The lack of sufficient virulence markers indicates that these EC isolates may be associated with asymptomatic infections. Similarly, the carriage of mainly chromosomally encoded virulence genes in CR-KP and CR-KV isolates suggested their possible association with asymptomatic carriage in healthy people or opportunistic infections in vulnerable individuals (Chapter 4) (Podschun & Ullmann, 1998; Holt et al., 2015). These asymptomatic infection-associated EC and *Klebsiella* isolates are likely to be underexplored by clinical surveillance focusing on symptomatic infections. Interestingly, some of these EC and *Klebsiella* isolates were also found to carry transferrable resistance genes. For instance, in some CR-EC isolates, evidence was found for the presence of plasmid-borne resistance genes against β-lactam ($bla_{\text{TEM-1B}}$ on IncM2 plasmid), quinolone (*qnrS2* on IncQ2), and sulfonamide (*sul2* on IncQ1) (Chapter 3). Likewise, some CR-KP and CR-KV isolates were also found to acquire β-lactam ($bla_{\text{TEM-1B}}$ on IncM2), aminoglycoside (*aac(3)-IId* on IncM2), and quinolone (*qnrS2* on IncQ2) resistance genes through plasmids (Chapter 4). Therefore, these asymptomatic infection-related CR isolates with plasmid-borne resistance genes could serve as underexplored reservoirs of transferrable resistance genes.

Taken together, the findings of Chapters 3 and 4 demonstrated the potential of wastewater-based surveillance to monitor the emergence and spread of resistance in non-clinical isolates and, in particular, isolates from the general community and non-human sources. With the ability to monitor resistance beyond clinical settings, routine wastewater-based surveillance can complement healthcare infection surveillance to

inform infection control and prevention strategies to effectively track and control the emergence and dissemination of antibiotic-resistant pathogens in the wider community. However, this study focused on wastewater isolates with unusual AMR patterns. Therefore, this study is limited because it does not describe the antibiotic resistance pattern of all wastewater isolates that could be subject to future studies.

5.3 Future perspectives

My proposed wastewater-based monitoring of antibiotic-resistant bacteria in the urban community can be implemented in regions with a centralised wastewater system. However, the implication of this approach in low- and middle-income countries with a relatively high prevalence of AMR (WHO, 2014; Hassan, 2020) and regional areas of developed countries with primarily on-site sanitation systems such as pit latrines and septic tanks would be practically challenging (Berende et al., 2017; WHO, 2019c). Several studies in such regions suggested collecting faecal sludges from shared latrines, multiple household on-site facilities, and disposal sites, such as major drains, ditches and canals that collect waste from many latrines and septic tanks, as a community sampling approach to monitor enteric pathogens (LaHue & Alexander, 2018; Capone et al., 2020), and recently for the prevalence of COVID-19 (Calabria de Araujo et al., 2021; Gwenzi, 2022). Therefore, it would be interesting to assess the feasibility of monitoring faecal sludges from on-site sanitation systems to better understand the emergence and dissemination of antibiotic-resistant pathogens in understudied communities without a centralised wastewater system.

This study applied a culture-based approach to isolate and enumerate antibiotic-resistant bacterial colonies that reveal their geospatial-temporal and demographic trends in the community, and Illumina short-read genome sequencing that identifies emerging and new strains of EC and *Klebsiella* and novel resistance mechanisms. Despite the precision and cost-effectiveness of short-read sequencing, the process is often lengthy (Liu et al., 2012; Hu et al., 2021; Khedher et al., 2022), limiting its ability to promptly warn of an unexpected rise in resistance in the community. Alternatively, faster, higher-throughput long-read sequencing technology, such as Oxford Nanopore Technology, is available that offers complete genome sequencing in several hours, but this technique is relatively expensive (Bradley et al., 2015; van Dijk et al., 2018; Gorzynski et al., 2022). Once

affordable, this technology can be used in future wastewater-based monitoring studies to rapidly identify emerging and novel antibiotic-resistant clones in the community. Recently, several studies proposed culture-independent methods for AMR surveillance, such as metagenomic sequencing of wastewater, that can quantify all resistance genes in a single sample and provide information on their trends and factors driving their occurrence and distribution in the community (Su et al., 2017; Hendriksen et al., 2019; Pärnänen et al., 2019; Karkman et al., 2020; Li et al., 2022). However, linking resistance gene information with specific bacterial strains is often challenging (Forbes et al., 2017; Oniciuc et al., 2018) and this limits the ability to identify novel bacterial strains and the introduction of emerging resistant clones into the community.

Although this thesis reveals the trends of antibiotic-resistant bacteria and identifies possible factors driving their distribution in wastewater catchments, the available data from only six-time points are insufficient to identify the characteristic pattern indicative of an outbreak in the community. With continuous monitoring of wastewater, more data will be collected that can be utilised by artificial intelligence and its related machine learning algorithms to extract patterns before outbreaks or detect deviations from general trends and predict any future outbreak to inform public services to respond quickly to effectively control the outbreak (Chae et al., 2018; Wong et al., 2019; Chiu et al., 2022). Recently, machine learning techniques have been used to predict COVID-19 outbreaks in the community with reasonable accuracy using WBE (Li et al., 2021; Abdeldayem et al., 2022; Jiang et al., 2022), further supporting the notion that the continuous wastewater-based monitoring approach can predict outbreaks of antibiotic-resistant bacteria in the community.

Although this study found the relatedness of ESBL-E and CRE wastewater isolates to human-associated clones, extensive evidence exists on their occurrence in diverse non-human sources, including animals such as wild, companion, livestock, poultry, avian, primate, food production; and the environment such as waterways and soils (Bélanger et al., 2013; Abraham et al., 2015; Woolhouse et al., 2015; Jamborova et al., 2018; Wyres & Holt, 2018; Barrios-Camacho et al., 2019; Mukerji et al., 2019; Hooban et al., 2020; Kidsley et al., 2020; Cherak et al., 2021; Wareth & Neubauer, 2021). These non-human sources have also been suggested as potential reservoirs for amplifying and transmitting

resistant organisms or genes across various niches (Bélanger et al., 2013; Wyres & Holt, 2018; Barrios-Camacho et al., 2019; Marques et al., 2019; Hooban et al., 2020). The present study also found evidence for the association of an ESBL-EC isolate from wastewater with a non-human-associated clone (ST9586). These observations underscore the need to implement a 'One Health' genomic epidemiological approach that connects human health with the health of animals and the environment to evaluate the spread of antibiotic resistance in non-human sources and the risk of their transmission to humans (Collignon, 2013; Robinson et al., 2016; McEwen & Collignon; 2018). Genomic-informed targeted surveillance of non-human sources, particularly retail food products (animal and plant origin) and waterways receiving wastewater effluents, could be used in the future along with wastewater-based and healthcare-infection surveillance to identify the genetic relatedness of antibiotic-resistant isolates from diverse niches to effectively track and control their emergence, dissemination, and transmission from non-human sources to humans, or vice versa.

Appendix A: Chapter 2 Supplementary Information

Text S2.1 Selection, confirmation, and enumeration of antibiotic-resistant bacterial colonies

All ChromID agars that were overlaid with wastewater samples yielded diverse coloured colonies. On the selective ChromID ESBL agars, a variety of pink, green, brown, and white colonies of different types of bacteria grew, of which the pink colonies were identified as members of the *Enterobacteriaceae* family by MALDI-TOF testing were from 16 locations in July 2017, from 20 sites in February 2018, from 23 locations in May, from 24 sites in September 2018, from 17 locations in February 2019, and from 22 sites in June 2019. Also, green colonies from a single location in July 2017, February 2018, and June 2019; and both pink and green colonies, on the same ESBL plate, from eight sites in July 2017, four locations in February 2018, two sites in May 2018, one site in September 2018, eight locations in February 2019, and two sites in June 2019 were detected as *Enterobacteriaceae* by MALDI-TOF. All the identified *Enterobacteriaceae* isolates were confirmed as ESBL producers by the VITEK 2 advanced expert system™ (AES) based on the analysis and interpretation of the MIC patterns of several β-lactam antibiotics (i.e. ampicillin, cefoxitin, cefazolin, ceftazidime, ceftriaxone, cefepime, and meropenem) and the β-lactams with β-lactamase inhibitors (i.e. amoxicillin/clavulanic acid, piperacillin/tazobactam, and ticarcillin/clavulanic acid) incorporated in the AST-N246 card. Since ChromID ESBL agar was found to be highly specific for ESBL-producing *Enterobacteriaceae* (ESBL-E) (Blaak et al., 2015), no further colony confirmation was done before enumeration. An estimate of the ESBL-E CFUs/ml of wastewater was obtained from the number of pink and green colonies on ChromID ESBL agar (either the 1:10 or 1:100 dilution), the dilution factor, and the volume plated.

Various pink, purple, green-blue, green, brown, and white colonies were found on the selective ChromID CARBA SMART plates. Among these, pink colonies were identified as *Enterobacteriaceae* by MALDI-TOF from three sites in July 2017 and one location in all other sampling periods. Green-blue colonies from four sites in July 2017, one location in May 2018, five sites in September 2018, and two locations in February 2019 and June 2019; purple colonies from one site in September 2018, one location in February 2019, and two sites in June 2019; and both purple and green-blue colonies from a single location

in September 2018 were also confirmed as *Enterobacteriaceae* by MALDI-TOF. VITEK 2 confirmed the identified *Enterobacteriaceae* colonies as CRE based on the elevated meropenem MIC (i.e. ≥ 4 μg/mL) following the Clinical and Laboratory Standards Institute (CLSI) guidelines **(**Clinical and Laboratory Standards Institute, 2018). All the confirmed CRE colonies mentioned here were from the CARB compartment (mainly *Klebsiella pneumoniae* carbapenemase (KPC)- and metallo-carbapenemase-types) of the CARBA SMART agar. In contrast, none of the colonies from the OXA compartment (OXA-48-type) of the CARBA SMART agar was confirmed as CRE by VITEK 2. This may be either because the colonies from the OXA compartment were non-carbapenemase producers or OXA-48-types, many of which were not resistant to meropenem (Oueslati et al., 2015). Therefore, they may not be identified as CRE by VITEK 2, which uses resistance against several β-lactams, including meropenem, to confirm carbapenemase production. An estimate of the CRE CFUs/ml of wastewater was obtained from the number of pink, green and purple colonies on the CARB compartment of ChromID CARBA SMART agar (either the 1:10 or 1:100 dilution), the dilution factor, and the volume plated.

ChromID VRE plates yielded several purple, violet, green, blue-green, yellow, brown, pink, and white colonies. Of which, purple colonies found on VRE plates were identified as *Enterococcus spp.* by MALDI-TOF testing from two, five, nine, eight, and four locations in July 2017, February 2018, May 2018, September 2018, and June 2019, respectively. Violet colonies from four sites in September 2018 and five locations in February 2019; green-blue colonies from one location in February 2019 and June 2019 were also identified as *Enterococcus spp.* by MALDI-TOF testing. VITEK 2 confirmed the identified enterococcal colonies as VRE based on the resistance patterns of vancomycin (i.e. MIC ≥ 32 μg/mL) and teicoplanin (i.e. MIC ≥ 32 μg/mL) antibiotics in the AST-P612 card, following the CLSI guidelines (Clinical and Laboratory Standards Institute, 2018). The VRE CFUs/ml of wastewater were calculated from the number of purple, violet and green-blue colonies on ChromID VRE agar, the dilution factor, and the volume plated.

Among the green, pink, blue-green, yellow, and white colonies on the ChromID MRSA plates, only green colonies were identified by MALDI-TOF as *Staphylococcus aureus*

from two sites in July 2017, one location in February 2018, a single site in May 2018, and two locations in September 2018 and February 2019. None of the green colonies was tested positive for MRSA isolates in June 2019. VITEK 2 AES confirmed the identified *S. aureus* as MRSA based on resistance against oxacillin (i.e. MIC ≥ 4 µg/mL) and positive cefoxitin screening incorporated in the AST-P612 card in accordance with the CLSI guidelines (Clinical and Laboratory Standards Institute, 2018). The MRSA CFUs/ml of wastewater were calculated from the number of green colonies on ChromID MRSA agar, the dilution factor, and the volume plated.

Text S2.2 Estimation of wastewater catchments population density

The population density of each wastewater catchment was calculated at the mesh block (MB) level in ArcMap following the method described elsewhere (Tscharke et al., 2019). MBs are the smallest geographical unit of the Australian Bureau of Statistics (ABS) that usually consists of fewer than 60 dwellings. The area of the MBs was downloaded as geospatial files (shapefile) from the ABS website (https://www.abs.gov.au/AUSSTATS/abs@.nsf/DetailsPage/1270.0.55.001July%202016?OpenDocument#Data). The usual resident population dataset of the 2016 Census was extracted at the MB level with 11-digit MB codes for New South Wales (NSW) using the online ABS table builder platform (ABS, 2016). The population data was then attached with the respective MBs using the Join tool in ArcMap. This new data layer with the MBs and the corresponding population was then overlaid with the wastewater catchment layer. As the wastewater catchment and the MBs boundary were not defined using the same geographical standard/criteria, some MBs/catchment overlap extended beyond the sewage catchment boundary (Figure S2.5). Where the MB/catchment boundary partially overlapped, the proportion of MBs that were within the wastewater catchment was calculated using the Tabulate Intersection tools in ArcMap. Then, the proportional population of these partially intersected MBs within the catchment boundary was estimated as:

(Number of people in MB × Percentage of MB within catchment boundary)/100.

Finally, all MBs that were fully and partially intersected with the wastewater catchment boundary were aggregated using the Spatial Join tool in ArcMap to calculate the total number of populations within each catchment. The total population of each catchment

was divided by the respective catchment area (square kilometre) to obtain the population density per square kilometre.

Text S2.3 Data source, categorisation, and geoprocessing of the healthcare infection-related variables

Public hospitals were classified into several peer groups by the Australian Institute of Health and Welfare (AIHW) based on the type and nature of provided services. Datasets with the address of the five major public hospital peer groups in NSW, such as principal referral, public acute group A, public acute group B, public acute group C, and public acute group D, were downloaded from the AIHW website (https://www.aihw.gov.au/reports/hospitals/australian-hospital-peer-groups/contents/table-of-contents). The public hospital dataset was geocoded in ArcMap based on the addresses and overlaid with the wastewater catchment boundary. The total number of public hospitals and peer groups within each catchment was then clipped and aggregated based on the wastewater catchment in ArcMap using the Clip and Spatial Join geoprocessing tools, respectively. The aggregated number of all public hospitals within each wastewater catchment comprises the variable number of public hospitals.

The NSW private hospital data were obtained from the MyHospitals Profile Dataset webpage on the AIHW website (http://www.myhospitals.gov.au/about-the-data/download-data). Based on the bed numbers, private hospitals were categorised into three size groups to facilitate further aggregation with similar-sized public hospitals. Private hospitals that fall into the first, second and third tertile were labelled as large (Q3, number of beds 200-500), medium (Q2, number of beds 50-199), and small (Q1, number of beds 0-50), respectively. The datasets with the number and categories of private hospitals (small, medium, and large) were geocoded based on the X and Y coordinates and aggregated based on the wastewater catchment area in ArcMap. The aggregated number of all small, medium and large private hospitals within each wastewater catchment encompasses the variable number of all private hospitals.

Based on bed numbers, the principal referral (average number of beds 653), public acute group A (average number of beds 279), and large private hospitals (number of beds 200-500) were combined into a new category for large hospitals. The total number of principal

referrals or public acute group A and large private (Q3) hospitals co-located within each wastewater catchment were aggregated to a new group of co-located large hospitals. Public acute group B, C and D hospitals were aggregated into smaller public hospitals. The medium and small private hospitals were aggregated to create a group of smaller private hospitals. In addition, the number of hospital admissions, number of acute bed days, the number of elective surgeries, and the average length of hospital stay in the last three months were collected at the NSW local health district (LHD) level from the Bureau of Health Information quarterly dataset (https://www.bhi.nsw.gov.au/BHI_reports/healthcare_quarterly) and aggregated based on the wastewater catchment area.

The location data for residential aged care facilities (RACFs) was collected from the GEN Aged Care Data webpage on the AIHW website (https://www.gen-agedcaredata.gov.au/resources/access-data/2019/september/aged-care-service-list-30-june-2019). The RACF dataset was geocoded and aggregated in ArcMap based on the X and Y coordinates and the wastewater catchments, respectively. The RACFs were classified into three categories based on the bed number tertile; large (Q3, number of beds 200-500), medium (Q2, number of beds 50-199), and small (Q1, number of beds 0-50). The number and location of general practitioners (GPs) in NSW were obtained from the National Health Services Directory (NHSD), 2018 dataset, published by Healthdirect Australia, accessed through the Australian Urban Research Infrastructure Network (AURIN) portal. The number of GPs was geocoded based on the X and Y coordinates in ArcMap and aggregated based on wastewater catchment areas.

Supplementary Tables

Table S2.1 Distribution of total and antibiotic-resistant bacterial load across 25 wastewater treatment plants (WWTPs) in six sampling periods.

WWTP	Sampling period	Log FNR ESBL-E load	Log FNR CRE load	Log FNR VRE load	Log FNR MRSA load	Log total bacteria CFU/day
Penrith	July 2017	5.92	ND*	ND	ND	13.84
Castle Hill	July 2017	6.64	ND	ND	ND	14.58
West Hornsby	July 2017	6.07	4.86	ND	ND	13.75
Warriewood	July 2017	5.22	3.88	ND	ND	14.67
North Head	July 2017	4.94	3.38	ND	ND	12.61
Winmalee	July 2017	5.80	ND	ND	ND	13.41
Quakers Hill	July 2017	5.44	ND	ND	ND	14.21
Rouse Hill	July 2017	6.11	ND	ND	6.03	13.90
Riverstone	July 2017	6.24	4.81	ND	6.59	14.80
Brooklyn	July 2017	7.81	ND	ND	ND	15.21
Hornsby Heights	July 2017	6.24	ND	6.05	ND	14.35
Richmond	July 2017	5.72	ND	ND	ND	15.13
North Richmond	July 2017	6.39	ND	ND	ND	15.58
Wallacia	July 2017	6.65	ND	ND	ND	15.12
Wollongong	July 2017	5.79	ND	4.19	ND	13.48
Picton	July 2017	6.44	ND	ND	ND	14.78
West Camden	July 2017	6.12	ND	ND	ND	13.75
Cronulla	July 2017	5.13	3.67	ND	ND	13.92
Malabar	July 2017	4.98	ND	ND	ND	12.38
St Marys	July 2017	5.60	ND	ND	ND	14.08
Bondi	July 2017	5.16	ND	ND	ND	13.25
Shellharbour	July 2017	5.40	ND	ND	ND	14.76
Bombo	July 2017	6.55	ND	ND	ND	14.20
Glenfield	July 2017	5.49	3.83	ND	ND	14.06
Liverpool	July 2017	6.25	4.64	ND	ND	13.19
Penrith	February 2018	3.93	ND	ND	ND	14.64
Castle Hill	February 2018	5.58	ND	ND	ND	14.49
West Hornsby	February 2018	5.14	ND	ND	ND	14.41
Warriewood	February 2018	5.11	ND	ND	ND	13.83
North Head	February 2018	4.14	ND	ND	ND	12.28
Winmalee	February 2018	4.98	ND	ND	ND	14.25
Quakers Hill	February 2018	5.09	ND	3.16	ND	13.67
Rouse Hill	February 2018	6.13	ND	ND	ND	13.74
Riverstone	February 2018	5.75	ND	ND	ND	14.63
Brooklyn	February 2018	6.41	ND	ND	ND	15.44
Hornsby Heights	February 2018	5.47	ND	ND	ND	14.75
Richmond	February 2018	5.62	ND	4.59	5.51	15.30
North Richmond	February 2018	6.38	ND	ND	ND	15.51
Wallacia	February 2018	5.10	ND	ND	ND	15.33
Wollongong	February 2018	5.84	ND	4.84	ND	12.32
Picton	February 2018	5.20	ND	ND	ND	15.03
West Camden	February 2018	4.89	ND	ND	ND	14.17
Cronulla	February 2018	5.15	3.96	4.63	ND	13.14
Malabar	February 2018	4.74	ND	ND	ND	11.68
St Marys	February 2018	4.59	ND	4.65	ND	13.73
Bondi	February 2018	4.77	ND	ND	ND	12.73
Shellharbour	February 2018	5.19	ND	ND	ND	13.71
Bombo	February 2018	5.45	ND	ND	ND	14.26
Glenfield	February 2018	3.90	ND	ND	ND	14.96

Liverpool	February 2018	5.14	ND	ND	ND	13.76
Penrith	May 2018	5.58	ND	ND	ND	13.35
Castle Hill	May 2018	6.57	ND	ND	ND	13.99
West Hornsby	May 2018	5.59	ND	ND	ND	14.08
Warriewood	May 2018	5.36	ND	4.69	ND	14.09
North Head	May 2018	4.44	ND	3.00	ND	12.31
Winmalee	May 2018	4.90	ND	ND	ND	13.71
Quakers Hill	May 2018	5.61	4.85	4.72	ND	13.54
Rouse Hill	May 2018	5.01	ND	ND	4.99	13.58
Riverstone	May 2018	6.62	ND	ND	ND	14.38
Brooklyn	May 2018	6.51	ND	ND	ND	15.40
Hornsby Heights	May 2018	5.51	ND	ND	ND	14.32
Richmond	May 2018	5.80	ND	ND	ND	14.08
North Richmond	May 2018	6.51	ND	ND	ND	14.84
Wallacia	May 2018	5.90	ND	ND	ND	14.80
Wollongong	May 2018	5.36	ND	6.04	ND	13.20
Picton	May 2018	5.61	ND	ND	ND	14.84
West Camden	May 2018	4.79	ND	5.17	ND	14.12
Cronulla	May 2018	5.88	ND	4.72	ND	13.43
Malabar	May 2018	5.44	ND	ND	ND	11.53
St Marys	May 2018	4.96	ND	5.72	ND	14.01
Bondi	May 2018	4.33	4.25	4.28	ND	13.07
Shellharbour	May 2018	5.69	ND	ND	ND	13.72
Bombo	May 2018	6.40	ND	ND	ND	14.19
Glenfield	May 2018	5.56	ND	ND	ND	13.51
Liverpool	May 2018	5.46	ND	4.98	ND	13.43
Penrith	September 2018	4.84	5.50	ND	ND	13.57
Castle Hill	September 2018	5.62	ND	ND	ND	13.83
West Hornsby	September 2018	5.31	ND	4.84	4.84	13.75
Warriewood	September 2018	5.11	3.98	ND	ND	14.08
North Head	September 2018	3.79	3.59	ND	ND	12.47
Winmalee	September 2018	5.14	ND	4.64	ND	13.75
Quakers Hill	September 2018	5.19	ND	ND	ND	13.44
Rouse Hill	September 2018	5.43	5.09	ND	4.79	13.41
Riverstone	September 2018	6.44	ND	6.07	ND	14.00
Brooklyn	September 2018	6.32	ND	7.10	ND	15.18
Hornsby Heights	September 2018	5.35	ND	ND	ND	14.44
Richmond	September 2018	7.05	ND	ND	ND	14.37
North Richmond	September 2018	6.16	ND	ND	ND	14.79
Wallacia	September 2018	6.35	ND	ND	ND	15.18
Wollongong	September 2018	5.28	ND	5.03	ND	13.21
Picton	September 2018	5.52	ND	4.92	ND	14.66
West Camden	September 2018	5.42	ND	4.34	ND	13.20
Cronulla	September 2018	4.76	ND	4.95	ND	13.26
Malabar	September 2018	4.02	3.72	ND	ND	12.13
St Marys	September 2018	5.10	ND	ND	ND	13.37
Bondi	September 2018	4.82	4.58	4.60	ND	12.65
Shellharbour	September 2018	5.41	ND	5.31	ND	13.76
Bombo	September 2018	5.40	ND	6.02	ND	14.28
Glenfield	September 2018	5.39	4.77	ND	ND	13.49
Liverpool	September 2018	5.08	4.61	4.21	ND	13.19
Penrith	February 2019	5.67	ND	ND	ND	13.51
Castle Hill	February 2019	6.35	ND	ND	ND	13.82
West Hornsby	February 2019	5.82	ND	ND	ND	13.68
Warriewood	February 2019	5.44	ND	ND	4.34	13.67
North Head	February 2019	3.25	ND	2.10	ND	12.79
Winmalee	February 2019	5.35	ND	4.31	ND	13.57
Quakers Hill	February 2019	5.05	ND	3.65	ND	13.63
Rouse Hill	February 2019	5.43	ND	ND	ND	13.73
Riverstone	February 2019	6.19	4.18	ND	ND	14.33

Brooklyn	February 2019	6.42	ND	ND	ND	15.14
Hornsby Heights	February 2019	5.60	ND	ND	ND	14.33
Richmond	February 2019	6.14	ND	ND	ND	14.86
North Richmond	February 2019	6.04	ND	ND	ND	15.00
Wallacia	February 2019	5.26	ND	ND	ND	15.37
Wollongong	February 2019	4.80	ND	ND	ND	13.30
Picton	February 2019	5.79	ND	ND	ND	14.68
West Camden	February 2019	5.46	ND	ND	ND	13.75
Cronulla	February 2019	4.88	ND	ND	ND	13.16
Malabar	February 2019	3.19	2.53	ND	ND	12.69
St Marys	February 2019	5.03	ND	4.37	ND	13.45
Bondi	February 2019	4.07	3.89	3.37	3.67	12.89
Shellharbour	February 2019	5.18	ND	ND	ND	13.82
Bombo	February 2019	5.40	ND	ND	ND	14.31
Glenfield	February 2019	3.86	2.77	ND	ND	14.59
Liverpool	February 2019	4.46	ND	3.24	ND	13.83
Penrith	June 2019	4.98	ND	ND	ND	13.56
Castle Hill	June 2019	5.69	ND	ND	ND	14.05
West Hornsby	June 2019	5.61	ND	5.53	ND	12.83
Warriewood	June 2019	5.31	5.01	ND	ND	13.01
North Head	June 2019	3.92	ND	ND	ND	12.15
Winmalee	June 2019	5.52	ND	ND	ND	13.08
Quakers Hill	June 2019	4.64	ND	ND	ND	13.60
Rouse Hill	June 2019	5.53	ND	ND	ND	13.52
Riverstone	June 2019	6.42	4.33	ND	ND	14.02
Brooklyn	June 2019	8.57	ND	7.25	ND	14.90
Hornsby Heights	June 2019	6.02	ND	ND	ND	13.94
Richmond	June 2019	5.73	ND	ND	ND	14.74
North Richmond	June 2019	6.69	ND	ND	ND	14.60
Wallacia	June 2019	5.83	ND	ND	ND	14.91
Wollongong	June 2019	4.68	ND	ND	ND	12.98
Picton	June 2019	5.70	ND	4.95	ND	14.70
West Camden	June 2019	5.40	4.66	ND	ND	13.51
Cronulla	June 2019	5.10	ND	ND	ND	12.70
Malabar	June 2019	3.94	ND	ND	ND	12.47
St Marys	June 2019	4.65	ND	ND	ND	13.77
Bondi	June 2019	5.17	4.57	ND	ND	11.87
Shellharbour	June 2019	4.51	ND	ND	ND	13.74
Bombo	June 2019	5.66	ND	4.66	ND	13.86
Glenfield	June 2019	5.48	4.12	3.94	ND	13.20
Liverpool	June 2019	5.56	ND	ND	ND	13.16

ND* = Not detected

Table S2.2 Spearman's rank correlation (r_s) analysis using $\log_{10}$ flow normalised relative (FNR) load of extended-spectrum β-lactamases-producing *Enterobacteriaceae* (ESBL-E) in wastewater as the outcome.

Variables	r_s value	*p* value
$\log_{10}$ population density per square kilometre	-0.47	<0.001
Proportion of the population between 19 to 50 years of age	-0.38	<0.001
Proportion of the population who had completed at least high school	-0.23	0.0047
Proportion of the population who had completed vocational education	0.38	<0.001
Number of schools per 10,000 population	0.4	<0.001
Square root of the number of small public hospitals per 10,000 population	0.45	0.0014
Average length of hospital stay in the last three months per 10,000 population	0.52	<0.001
$\log_{10}$ number of elective surgeries in the last three months per 10,000 population	0.25	0.032
Number of all RACFs per 10,000 population	0.49	<0.001
Number of large-sized RACFs per 10,000 population	0.49	<0.001
Number of medium-sized RACFs per 10,000 population	0.4	<0.001
$\log_{10}$ number of GPs per 10,000 population	0.23	0.0054

Table S2.3 Spearman's rank correlation (r_s) analysis using $\log_{10}$ flow normalised relative (FNR) load of carbapenem-resistant *Enterobacteriaceae* (CRE) in wastewater as the outcome.

Variables	r_s value	*p* value
Proportion of the population between 19 to 50 years of age	-0.28	0.04
Number of all hospitals per 10,000 population	-0.35	0.01
Square root of the number of small public hospitals per 10,000 population	0.72	0.018
Average length of hospital stay in the last three months per 10,000 population	0.78	<0.001
Number of co-located large hospitals per 10,000 population	0.82	0.012

Table S2.4 Spearman's rank correlation (r_s) analysis using $\log_{10}$ flow normalised relative (FNR) load of vancomycin-resistant enterococci (VRE) in wastewater as the outcome.

Variables	r_s value	*p* value
$\log_{10}$ population density per square kilometre	-0.47	0.0025
Proportion of the population between 19 to 50 years of age	-0.48	0.0018
Proportion of the population who had completed at least high school	-0.27	0.01
Proportion of the population who had completed vocational education	0.4	0.012
Number of schools per 10,000 population	0.45	0.0039
Number of co-located large hospitals per 10,000 population	0.87	0.05

Supplementary Figures

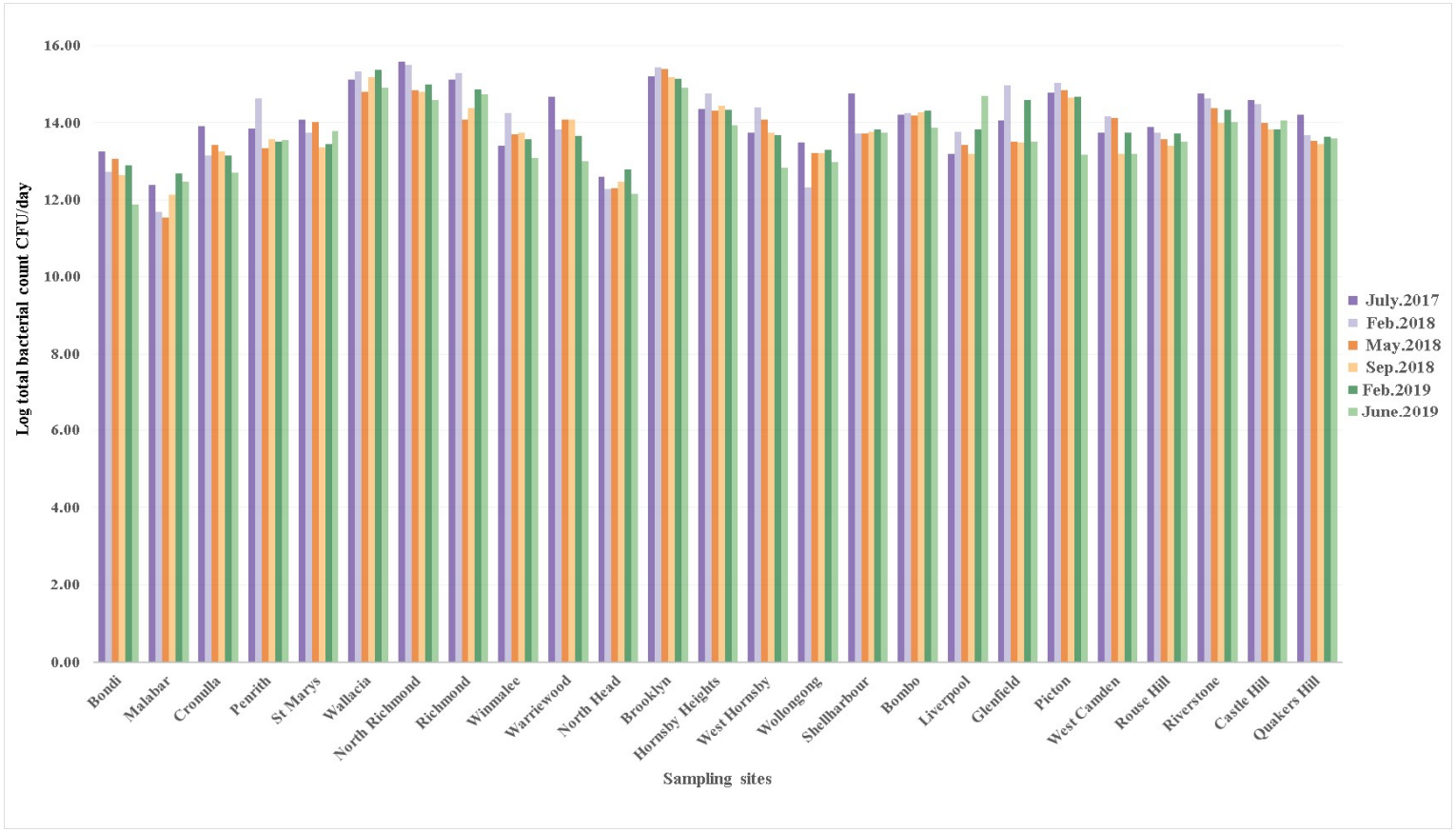


Figure S2.1 Distribution of total bacterial isolates across 25 wastewater treatment plants (WWTPs) in six sampling periods.

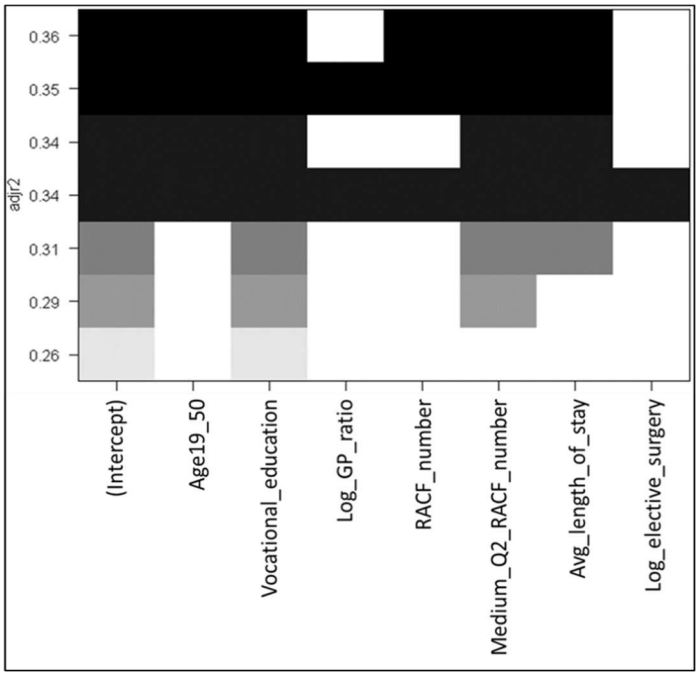


Figure S2.2 The best seven models for each subset size, based on the adjusted R-square for the outcome $\log_{10}$ FNR ESBL-E load. The graph suggests that the five-predictor model (top row) with the proportion of the population between 19 to 50 years of age, the proportion of the population who had completed vocational education, number of all RACFs per 10,000 population, number of medium-sized RACFs per 10,000 population, and the average length of hospital stay in the last three months is the best.

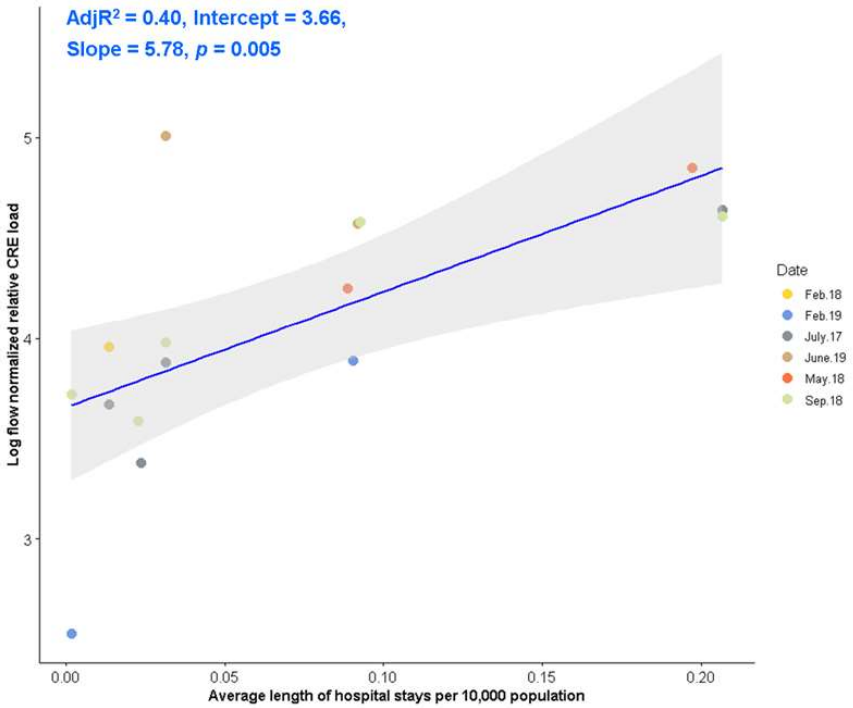


Figure S2.3 Simple linear regression analysis showing a significant positive association between $\log_{10}$ FNR CRE load (intercept = 3.66) in wastewater and the average length of hospital stay in the last three months ($\beta = 5.78$, $p = 0.005$) with an adjusted R^2 (i.e. coefficient of determination) value of 0.40.

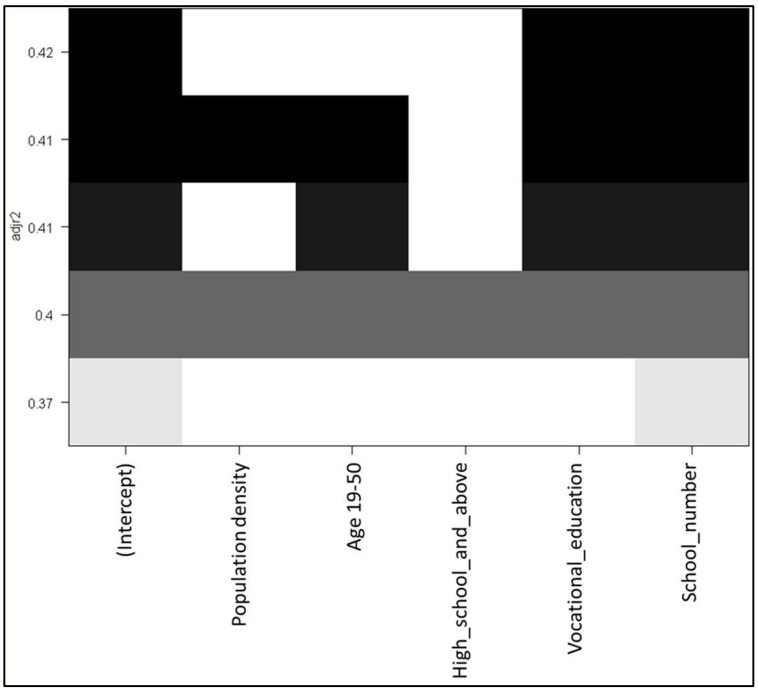


Figure S2.4 The best five models for each subset size, based on the adjusted R-square for the outcome $\log_{10}$ FNR VRE load. The graph suggests that the two-predictor model (top row) with the proportion of the population who had completed vocational education and the number of schools per 10,000 population is the best.

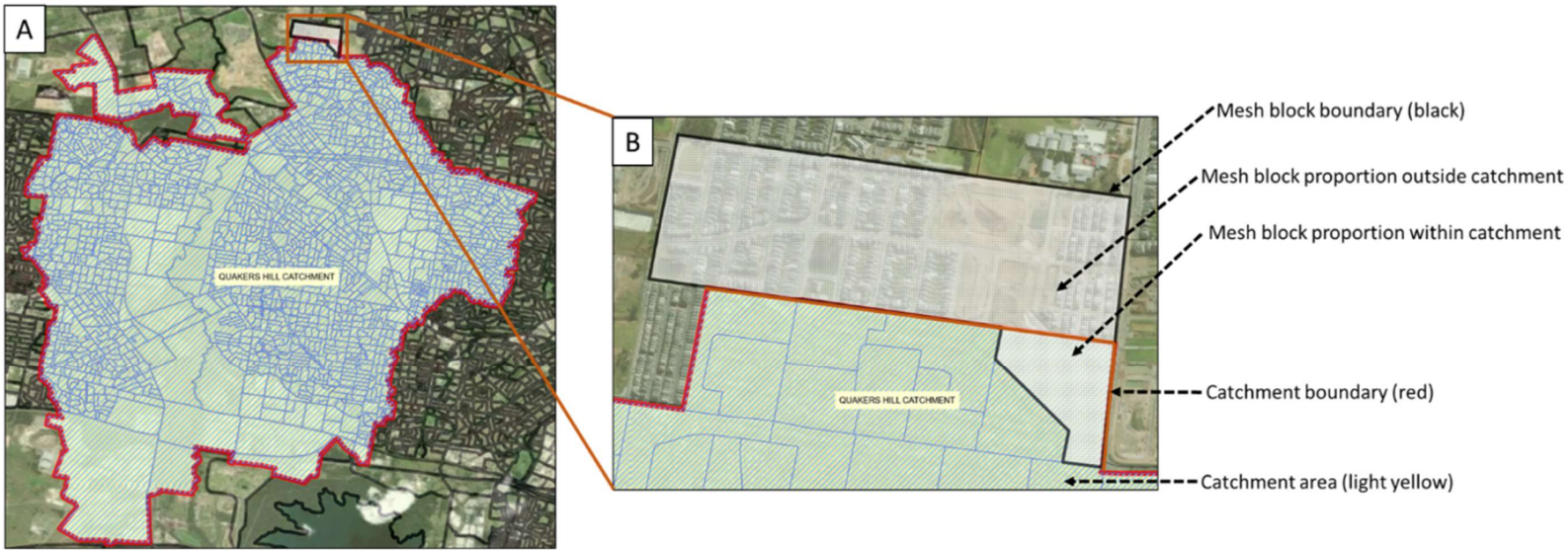


Figure S2.5 A) Illustration of a catchment boundary (red) overlaid with a mesh block boundary (black). B) An example of an intersecting mesh block with the catchment boundary. The proportion of a mesh block within a catchment area was used to estimate the proportional population of that intersecting mesh block. The total population within a catchment was calculated by aggregating all the mesh blocks (entirely or partially) intersecting with the catchment boundary.

Appendix B: Chapter 3 Supplementary Information

Supplementary Tables

Table S3.1 Antibiotic susceptibility pattern of the carbapenem-resistant *E. coli* (CR-EC) (n = 12) and extended-spectrum β-lactamases-producing *E. coli* (ESBL-EC) (n = 151) isolated from wastewater-based surveillance in Sydney at six-time points between 2017 and 2019. Isolates selected for whole-genome sequencing are in bold and highlighted in orange for resistant (R), blue for intermediate susceptible (I), and green for susceptible isolates (S).

				Antibiotic Classes																		
				β-lactams							Penicillins with β-lactamase inhibitors			Aminoglycosides			Fluoroquinolones		Dihydrofolate reductase inhibitors	Combinations	Nitrofuran derivatives	Phosphonic acid derivatives
				Aminopenicillin	Cephamycin	Cephalosporins				Carbapenem												
Sequenced Genomes	Month and year of isolation	Wastewater catchments	Phenotypes	Ampicillin	Cefoxitin	Cefazolin	Ceftazidime	Ceftriaxone	Cefepime	Meropenem	Amoxicillin/clavulanic acid	Piperacillin/tazobactam	Ticarcillin/clavulanic acid	Gentamicin	Tobramycin	Amikacin	Ciprofloxacin	Norfloxacin	Trimethoprim	Trimethoprim-Sulfamethoxazole	Nitrofurantoin	Fosfomycin
G4	**July 2017**	**West Hornsby**	**CR-EC**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**I**	**S**
G5	**July 2017**	**Riverstone**	**CR-EC**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**I**	**S**
G8	**May 2018**	**Bondi**	**CR-EC**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**S**
G16	**February 2019**	**Malabar**	**CR-EC**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**R**	**I**	**S**
	February 2018	Cronulla	CR-EC	R	R	R	S	R	S	R	R	R	S	S	S	S	R	R	R	R	S	S
	May 2018	Quakers Hill	CR-EC	R	R	R	R	R	R	R	R	R	S	S	I	S	R	R	R	R	S	S
	September 2018	Bondi	CR-EC	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	R	R	S	S
	September 2018	Rouse Hill	CR-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	S	S	R	R	S	S
	February 2019	Riverstone	CR-EC	R	R	R	R	R	R	R	R	R	R	S	R	S	R	R	R	R	I	S
	June 2019	Riverstone	CR-EC	R	R	R	R	R	R	R	R	R	R	S	R	S	R	R	R	R	I	S
	June 2019	West Camden	CR-EC	R	R	R	R	R	R	R	R	R	R	R	I	S	R	R	S	S	S	S
	June 2019	Warriewood	CR-EC	R	R	R	R	R	R	S	R	R	R	R	S	S	R	S	R	R	R	S
G10	**September 2018**	**Warriewood**	**ESBL-EC**	**R**	**R**	**R**	**R**	**R**	**R**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**I**	**S**	**S**	**S**	**R**	**S**
G11	**September 2018**	**Warriewood**	**ESBL-EC**	**R**	**R**	**R**	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**S**	**S**	**S**	**R**	**S**	**S**	**S**	**R**	**S**
G15	**February 2019**	**Winmalee**	**ESBL-EC**	**R**	**R**	**R**	**R**	**R**	**R**	**S**	**S**	**S**	**S**	**R**	**I**	**S**	**S**	**S**	**R**	**R**	**R**	**I**
G17	**June 2019**	**Warriewood**	**ESBL-EC**	**R**	**R**	**R**	**R**	**R**	**R**	**S**	**R**	**I**	**R**	**R**	**S**	**S**	**R**	**S**	**R**	**R**	**R**	**S**

July 2017	Wallacia	ESBL-EC	R	S	R	R	R	R	S	I	S	S	R	R	S	R	R	R	R	S	S
July 2017	West Camden	ESBL-EC	R	S	R	S	R	S	S	S	S	S	R	R	S	R	R	R	R	S	S
July 2017	Wollongong	ESBL-EC	R	R	R	R	R	S	S	R	S	S	S	I	S	S	S	R	R	S	S
July 2017	Winmalee	ESBL-EC	R	I	R	R	R	R	S	S	S	S	S	S	S	R	R	S	S	S	S
July 2017	Liverpool	ESBL-EC	R	S	R	R	R	S	S	I	S	S	S	R	S	S	S	R	R	S	S
July 2017	Richmond	ESBL-EC	R	S	R	S	R	S	S	S	S	S	R	I	S	S	S	R	R	S	S
July 2017	Hornsby Heights	ESBL-EC	R	S	R	S	R	S	S	S	S	S	R	S	S	S	S	R	R	S	S
July 2017	Warriewood	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S
July 2017	Bombo	ESBL-EC	R	I	R	R	R	S	S	R	S	S	S	S	S	S	S	S	S	S	S
July 2017	Brooklyn	ESBL-EC	R	S	R	S	R	S	S	R	I	S	S	S	S	S	S	S	S	S	S
July 2017	Bondi	ESBL-EC	R	S	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
July 2017	Picton	ESBL-EC	R	S	R	R	R	R	S	R	R	S	S	S	S	S	S	R	R	S	S
July 2017	North Richmond	ESBL-EC	R	S	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
July 2017	Quakers Hill	ESBL-EC	R	I	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
July 2017	Malabar	ESBL-EC	R	S	R	S	R	S	S	S	S	S	S	S	S	S	S	R	R	S	S
July 2017	St Marys	ESBL-EC	R	I	R	S	R	S	S	S	S	S	S	S	S	S	S	R	R	S	S
July 2017	North Head	ESBL-EC	R	S	R	S	R	S	S	S	S	S	S	S	S	S	S	R	R	S	S
July 2017	Penrith	ESBL-EC	R	S	R	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
July 2017	Glenfield	ESBL-EC	R	S	R	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
July 2017	Rouse Hill	ESBL-EC	R	S	R	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
July 2017	Riverstone	ESBL-EC	R	S	R	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
July 2017	Castle Hill	ESBL-EC	R	S	R	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
July 2017	West Hornsby	ESBL-EC	R	S	R	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
July 2017	Cronulla	ESBL-EC	R	S	R	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
February 2018	Wollongong	ESBL-EC	R	R	R	R	R	R	R	R	R	R	S	S	S	R	R	R	R	S	S
February 2018	Shellharbour	ESBL-EC	R	S	R	R	R	S	S	I	S	I	S	S	S	I	S	S	S	I	S
February 2018	Penrith	ESBL-EC	R	S	R	R	R	R	S	S	S	S	R	I	S	S	S	R	R	I	S
February 2018	Warriewood	ESBL-EC	R	S	R	R	R	R	S	S	S	S	R	I	S	R	R	R	R	S	S
February 2018	Bondi	ESBL-EC	R	S	R	S	R	S	S	S	S	S	S	S	S	R	R	S	S	S	S
February 2018	Bombo	ESBL-EC	R	S	R	R	R	R	S	R	S	R	S	S	S	R	R	S	S	S	S
February 2018	West Hornsby	ESBL-EC	R	R	R	R	R	S	S	R	S	S	R	I	S	S	S	R	R	S	S
February 2018	West Camden	ESBL-EC	R	S	R	S	R	S	S	R	S	S	S	S	S	I	S	R	R	S	S
February 2018	St Marys	ESBL-EC	R	S	R	S	R	S	S	S	S	S	S	S	S	S	S	R	R	S	S
February 2018	North Richmond	ESBL-EC	R	S	R	S	R	S	S	S	S	S	S	S	S	S	S	R	R	S	S
February 2018	Richmond	ESBL-EC	R	S	R	S	R	S	S	S	S	S	S	S	S	S	S	R	R	S	S
February 2018	Riverstone	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	I	S	R	R	S	S
February 2018	Quakers Hill	ESBL-EC	R	S	R	R	R	R	S	R	I	R	R	I	S	S	S	R	R	S	S
February 2018	North Head	ESBL-EC	R	I	R	R	R	R	S	I	S	R	R	I	S	S	S	R	R	S	S
February 2018	Picton	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	I	S	R	R	S	S
February 2018	Liverpool	ESBL-EC	R	S	R	S	R	S	S	S	S	S	R	I	S	S	S	S	S	S	S

February 2018	Glenfield	ESBL-EC	R	S	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
February 2018	Malabar	ESBL-EC	R	S	R	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
February 2018	North Head	ESBL-EC	R	S	R	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
February 2018	Castle Hill	ESBL-EC	R	S	R	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
February 2018	Rouse Hill	ESBL-EC	R	S	R	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
May 2018	Liverpool	ESBL-EC	R	R	R	R	R	I	R	R	R	S	S	S	S	R	R	R	R	S	S
May 2018	Bondi	ESBL-EC	R	R	R	R	R	I	S	R	I	S	R	S	S	R	R	R	R	I	S
May 2018	Bombo	ESBL-EC	R	R	R	S	R	S	S	I	S	S	R	I	S	R	R	R	R	S	S
May 2018	Riverstone	ESBL-EC	R	R	R	R	R	S	S	R	S	S	S	S	S	S	S	R	R	S	S
May 2018	Picton	ESBL-EC	R	R	R	R	R	S	S	R	S	S	R	I	S	S	S	R	R	S	S
May 2018	Malabar	ESBL-EC	R	R	R	R	R	R	S	R	I	S	S	S	S	S	S	S	S	S	S
May 2018	Rouse Hill	ESBL-EC	R	S	R	R	R	I	S	I	S	S	R	R	S	R	R	S	S	S	S
May 2018	North Head	ESBL-EC	R	S	R	R	R	S	S	S	S	S	S	S	S	R	R	S	S	S	S
May 2018	West Camden	ESBL-EC	R	S	R	R	R	S	S	S	S	S	S	S	S	R	R	S	S	S	S
May 2018	Rouse Hill	ESBL-EC	R	S	R	R	R	S	S	I	S	S	S	S	S	R	R	S	S	S	S
May 2018	Hornsby Heights	ESBL-EC	R	S	R	R	R	S	S	S	S	S	S	S	S	R	R	R	R	I	S
May 2018	West Hornsby	ESBL-EC	R	S	R	S	R	S	S	I	S	S	S	S	S	R	R	R	R	S	S
May 2018	Shellharbour	ESBL-EC	R	S	R	S	R	S	S	S	S	S	S	S	S	R	R	R	R	S	S
May 2018	North Richmond	ESBL-EC	R	S	R	R	R	S	S	S	S	S	S	S	S	S	S	R	R	S	S
May 2018	Wollongong	ESBL-EC	R	I	R	S	R	S	S	S	S	S	S	S	S	S	S	R	R	I	S
May 2018	St Marys	ESBL-EC	R	S	R	S	R	S	S	S	S	S	S	S	S	S	S	R	S	S	S
May 2018	Brooklyn	ESBL-EC	R	S	R	S	R	S	S	S	S	S	R	I	S	S	S	S	S	S	S
May 2018	Cronulla	ESBL-EC	R	S	R	R	R	I	S	S	S	S	S	S	S	S	S	S	S	S	S
May 2018	Glenfield	ESBL-EC	R	S	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
May 2018	Penrith	ESBL-EC	R	I	R	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
May 2018	Wallacia	ESBL-EC	R	S	R	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
May 2018	Richmond	ESBL-EC	R	S	R	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
May 2018	Winmalee	ESBL-EC	R	S	R	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
May 2018	Warriewood	ESBL-EC	R	S	R	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
September 2018	Penrith	ESBL-EC	R	S	R	R	R	S	R	S	R	I	R	S	R	S	S	S	S	S	S
September 2018	West Hornsby	ESBL-EC	R	R	R	R	R	R	S	R	S	R	S	R	S	R	R	R	R	I	S
September 2018	Riverstone	ESBL-EC	R	S	R	R	R	S	S	I	S	I	S	R	S	R	R	R	R	S	S
September 2018	Quakers Hill	ESBL-EC	R	S	R	R	R	R	S	S	S	S	R	I	S	R	R	R	R	S	S
September 2018	St Marys	ESBL-EC	R	R	R	R	R	R	S	S	S	I	S	S	S	R	R	S	S	S	S
September 2018	Hornsby Heights	ESBL-EC	R	R	R	R	R	R	S	S	S	S	S	S	S	R	R	S	S	I	S
September 2018	Glenfield	ESBL-EC	R	R	R	R	R	R	S	R	S	S	S	S	S	S	S	R	S	S	S
September 2018	Cronulla	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	R	R	R	R	S	S
September 2018	Liverpool	ESBL-EC	R	I	R	R	R	R	S	S	S	I	S	S	S	R	R	R	R	I	S
September 2018	Wollongong	ESBL-EC	R	S	R	R	R	R	S	S	S	S	R	I	S	R	R	S	S	S	S
September 2018	Wallacia	ESBL-EC	R	S	R	R	R	R	S	S	S	S	R	S	S	S	S	R	S	S	S

September 2018	Brooklyn	ESBL-EC	R	S	R	R	R	R	S	I	S	I	R	I	S	S	S	R	R	S	S
September 2018	Picton	ESBL-EC	R	S	R	R	R	R	S	I	S	I	R	S	S	S	S	R	R	S	S
September 2018	Penrith	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	S	S	R	R	S	S
September 2018	North Richmond	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	S	S	R	R	S	S
September 2018	Richmond	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	I	S	R	R	S	S
September 2018	Malabar	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S
September 2018	Bondi	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S
September 2018	Winmalee	ESBL-EC	R	S	R	R	R	R	S	I	S	I	S	S	S	S	S	S	S	S	S
September 2018	North Head	ESBL-EC	R	S	R	R	R	R	S	I	S	I	S	S	S	S	S	S	S	S	S
September 2018	Shellharbour	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S
September 2018	Bombo	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S
September 2018	West Camden	ESBL-EC	R	S	R	R	R	R	S	S	S	I	S	I	S	S	S	S	S	S	S
September 2018	Rouse Hill	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S
February 2019	Riverstone	ESBL-EC	R	R	R	R	R	R	S	R	S	R	R	R	S	R	R	R	R	R	S
February 2019	North Richmond	ESBL-EC	R	S	R	R	R	R	S	S	S	S	R	I	S	R	R	R	R	S	S
February 2019	Winmalee	ESBL-EC	R	I	R	R	R	R	S	I	S	I	R	I	S	R	S	R	R	S	S
February 2019	Cronulla	ESBL-EC	R	S	R	R	R	R	S	S	S	S	R	I	S	R	S	R	R	S	S
February 2019	Richmond	ESBL-EC	R	S	R	R	R	R	S	R	S	S	S	S	S	R	R	R	R	S	S
February 2019	Richmond	ESBL-EC	R	S	R	R	R	R	S	R	S	S	S	S	S	R	R	R	R	S	S
February 2019	Castle Hill	ESBL-EC	R	R	R	R	R	R	S	R	I	R	S	S	S	R	R	R	R	S	S
February 2019	Picton	ESBL-EC	R	S	R	R	R	R	S	S	I	S	S	S	S	R	R	R	R	S	S
February 2019	Bondi	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	S	S	R	S	S	S
February 2019	North Head	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	R	S	R	R	S	S
February 2019	Brooklyn	ESBL-EC	R	R	R	R	R	R	S	R	S	S	S	S	S	R	S	R	R	S	S
February 2019	West Hornsby	ESBL-EC	R	S	R	R	R	R	S	R	S	R	S	S	S	R	S	R	R	S	S
February 2019	Warriewood	ESBL-EC	R	S	R	R	R	R	S	S	S	I	S	S	S	R	R	S	S	S	S
February 2019	Rouse Hill	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	R	R	S	S	S	S
February 2019	Malabar	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	I	S	R	R	S	S
February 2019	Malabar	ESBL-EC	R	S	R	R	R	R	S	I	S	I	S	S	S	S	S	R	R	S	S
February 2019	Wollongong	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	S	S	R	R	S	S
February 2019	Penrith	ESBL-EC	R	S	R	R	R	R	S	I	S	I	S	S	S	S	S	R	R	I	S
February 2019	Hornsby Heights	ESBL-EC	R	S	R	R	R	R	S	I	S	I	S	S	S	S	S	R	R	S	S
February 2019	Hornsby Heights	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	S	S	R	R	S	S
February 2019	Liverpool	ESBL-EC	R	S	R	R	R	R	S	I	S	I	S	S	S	S	S	R	R	S	S
February 2019	Wallacia	ESBL-EC	R	S	R	R	R	R	S	S	S	S	R	I	S	I	S	S	S	S	S
February 2019	St Marys	ESBL-EC	R	S	R	R	R	R	S	R	R	R	S	S	S	S	S	S	S	S	S
February 2019	Warriewood	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S
February 2019	Shellharbour	ESBL-EC	R	S	R	R	R	R	S	I	S	I	S	S	S	S	S	S	S	S	S
February 2019	Bombo	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S
February 2019	Penrith	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S

February 2019	Quakers Hill	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S
February 2019	St Marys	ESBL-EC	R	S	R	R	R	R	S	S	S	I	S	S	S	S	S	S	S	S	S
February 2019	West Camden	ESBL-EC	R	S	R	R	R	R	S	S	S	I	S	S	S	S	S	S	S	S	S
June 2019	Shellharbour	ESBL-EC	R	S	R	R	R	R	S	I	S	I	R	I	S	R	R	S	S	S	S
June 2019	West Camden	ESBL-EC	R	R	R	R	R	R	S	R	R	R	S	S	S	R	R	S	S	S	S
June 2019	North Head	ESBL-EC	R	S	R	R	R	R	S	I	S	I	S	S	S	R	R	S	S	S	S
June 2019	Castle Hill	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	R	R	S	S	S	S
June 2019	Hornsby Heights	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	R	S	R	R	S	S
June 2019	Winmalee	ESBL-EC	R	S	R	R	R	R	S	I	S	I	R	I	S	I	S	R	R	S	S
June 2019	Picton	ESBL-EC	R	S	R	R	R	R	S	S	S	S	R	I	S	S	S	R	R	S	S
June 2019	Malabar	ESBL-EC	R	S	R	R	R	R	S	R	R	R	R	R	S	S	S	R	R	S	S
June 2019	Wallacia	ESBL-EC	R	R	R	R	R	R	S	R	R	R	S	S	S	S	S	R	R	S	S
June 2019	Glenfield	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	S	S	R	R	S	S
June 2019	St Marys	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	S	S	R	R	S	S
June 2019	Bondi	ESBL-EC	R	S	R	R	R	R	S	S	S	I	S	S	S	R	S	S	S	S	S
June 2019	Richmond	ESBL-EC	R	I	R	R	R	R	S	R	S	I	S	S	S	S	S	S	S	S	S
June 2019	Liverpool	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S
June 2019	North Richmond	ESBL-EC	R	I	R	R	R	R	S	R	S	I	S	S	S	S	S	S	S	S	S
June 2019	Brooklyn	ESBL-EC	R	R	R	R	R	R	S	R	R	R	S	S	S	S	S	S	S	S	S
June 2019	West Hornsby	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S
June 2019	Cronulla	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S
June 2019	Quakers Hill	ESBL-EC	R	R	R	R	R	R	S	R	I	R	S	S	S	S	S	S	S	S	S
June 2019	Penrith	ESBL-EC	R	R	R	R	R	R	S	R	S	R	S	S	S	S	S	S	S	S	S
June 2019	Bombo	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S
June 2019	Rouse Hill	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S
June 2019	Riverstone	ESBL-EC	R	S	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S
June 2019	Wollongong	ESBL-EC	R	S	R	R	R	R	S	S	I	S	S	S	S	S	S	S	S	S	S
Categories of important antibiotics for human medicine (WHO, 2019b)			Critically important (High priority)	Highly important	Highly important	Critically important (Highest priority)	Critically important (Highest priority)	Critically important (Highest priority)	Critically important (High priority)	Critically important (High priority)	Critically important (High priority)	Not available	Critically important (High priority)	Critically important (High priority)	Critically important (High priority)	Critically important (Highest priority)	Critically important (Highest priority)	Highly important	Highly important	Important	Critically important (High priority)

Table S3.2 General characteristics of the assembled *E. coli* genomes.

Genomes	Number of reads after adapter and quality trimming	Number of contigs	N50 (Kbp)	Approximate genome size (Mbp)	G + C content (%)	Number of predicted genes	Number of predicted protein coding sequences (CDS)
G4	372,537	469	76.68	6.03	51.45	5,951	5,882
G5	662,002	270	60.83	6.13	51.81	6,002	5,943
G8	627,021	115	130.61	5.00	50.58	4,742	4,676
G16	388,952	95	191.17	4.82	50.53	4,562	4,498
G10	592,009	93	134.57	4.87	50.81	4,638	4,573
G11	653,256	86	198.10	5.07	50.46	4,790	4,721
G15	830,124	73	213.40	5.02	50.56	4,729	4,664
G17	593,192	116	191.08	5.10	50.52	4,826	4,759

Table S3.3 Analysis of mutations identified in OmpC porin of *E. coli* isolates from wastewater.

Isolates	Protein	Functional impact of mutations predicted by PROVEAN	
		Neutral	Deleterious
G4, G5, G8	OmpC	V24I, K48D, D49S, V50K, Q54K, S85E, A86P, N88S, E89D, N91S, G137D, N176S, P177A, S178H, F182M, S184T, N189G, A193V, L194F, R195E, G216A, I218V, A226D, T229N, A230T, A231G, Y232L, N235T, Q346R, N357D, deletions of GVT at positions 185-187 (del G-T (185-T187))	N165D, G190R, R191D, D208N, D225W
G16	OmpC	N176S, P177V, V186M, T229S, A230P, A231L, L296V, G309N, del FTSG (182-185) and insertions of GTIA between positions 306-307 (Ins GTIA (182-185))	D192G, R195L
G10	OmpC	D49S, V50E, M57V, S85T, A86S, E89K, N90E, F149Y, N176S, P177V, V186M, G216A, I218V, A226D, T229F, A230E, A231R, I233L, L296V, G309N, Ins D (87-88), del FTSG (182-185), Ins VVAG (307-308)	D192G, Ins GLNGYG (229-230)
G11, G15, G17	OmpC	D49S, V50E, N176S, P177V, S178D, V186M, G216A, I218V, T229F, A230E, A231R, I233L, L296V, G309N, Q346R, N357D, del FTSG (182-185), Ins VING (307-308)	D192G, Ins GLNGYG (229-230)

Table S3.4 Functional impact of mutations identified in regulatory proteins of multidrug-resistant (MDR) efflux pumps of wastewater *E. coli* isolates.

Isolates	Protein	Functional impact of mutations predicted by PROVEAN	
		Neutral	**Deleterious**
G4, G5, G8	AcrR	E8R, Q10A, E11R, V19C, L23S	A2G, R3T, T5N, K6Q, Q7T, A9S, E11R, T12N, R13A, Q14P, H15T, I16H, L17P, D18R, A20G, L21S, R22T
G10	EvgA	K69S, R70A, Q71N, Y72I, S73A, I75L, I76L, I77L, I78L, D84T, H85I, K89N, H90I, A92L, D93M	R68G, G74E, V79S, S80P, A81L, N83M, Y87T, C91V, A94L, G95A, A96L, N97M, G98D, F99S, deletions of V-G at positions 100-204 (del V-G (100-204))
G10	EvgS	I7F, L42V, R78Q, N133S, H205Q, N303D, S359N, L380S, E818D, G856V, V945I, Q951H	None

Table S3.5 List of contigs co-harbouring antibiotic resistance and virulence genes in the assembled genome of *E. coli* isolates from wastewater.

Isolates	Contig (size bp)	Plasmid replicon (position in contig)	Resistance gene (position in contig)	Virulence gene (position in contig)
G4	Node_74 (9606)	IncQ2 (4852 to 5301)	*qnrS2* (2564 to 3220)	Not detected (ND)
G5	Node_27 (61430)	IncM2 (1921 to 2584)	$bla_{\text{TEM-1B}}$ (60585 to 61355)	ND
G16	Node_51 (4540)	IncQ1 (3940 to 4468)	*sul2* (1786 to 2601)	ND
G10	Node_56 (10794)	IncFIB(AP001918) (1719 to 2400)	ND	*hlyF* (5454 to 6563), *ompT* (6996 to 7949)
G11	Node_42 (10524)	Col156 (6753 to 6894)	ND	*senB* (948 to 2123)
G15	Node_31 (19297)	IncFII(29) (6175 to 6433)	$bla_{\text{TEM-1B}}$ (313 to 1173)	ND
	Node_45 (10524)	Col156 (6753 to 6894)	ND	*senB* (948 to 2123)
G17	Node_43 (10524)	Col156 (3631 to 3772)	ND	*senB* (8402 to 9577)

Table S3.6 Impact of mutations in genes encoding proteins related to survival and motility of wastewater *E. coli* isolates.

Isolates	Protein	Functional impact of mutations predicted by PROVEAN	
		Neutral	**Deleterious**
G4	NfrA	A557T, T603A	Del A-L (598-602)
G11	NfrA	E393G, S511N, A533T, K573Q, S578N, V628I, P671Q, I784L, I835L	Del M-T (1-308)
G16	NfrA	R101Q, I274N, A357P, A364S, S511N, K573Q, S578N, N600S, T648I, P671Q, I835L	N645T
G4, G5, G8	Cir	D95Q, S96Q	S90Y, I91S, R92W, G93S, L94G, S97L, Y98H, T99P, L100D, I101S, L102R, V103R, D104R, del K-F (106-663)
G10	Cir	G161R, Q162S, K163E, S165V, T169Y, V170R, T172Y, R178S, D179R, R180S	I160N, W164M, T167Y, V168R, D171R, T173H, I174H, Q175S, E176G, H177T, G181R, del D-F (182-663)
G4, G5, G8	FlhA	L30M	Del M-L (1-29), I31S, L32N, S33L, M34A, M35A, V36M, P38R
G4, G5, G8	FliI	Q45R	L46N, P47L, L48C, G49H, del A-S (50-457)
G10	FliP	I217L	A215P, T216P, A218L, L219C, F221L, K222N, del L-S (223-245)

Supplementary Figures

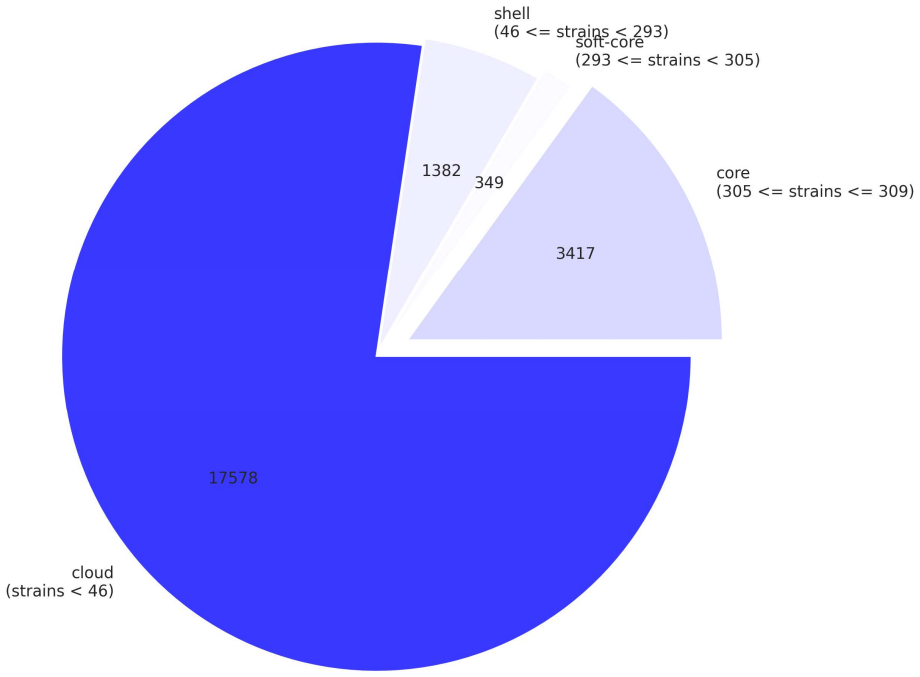
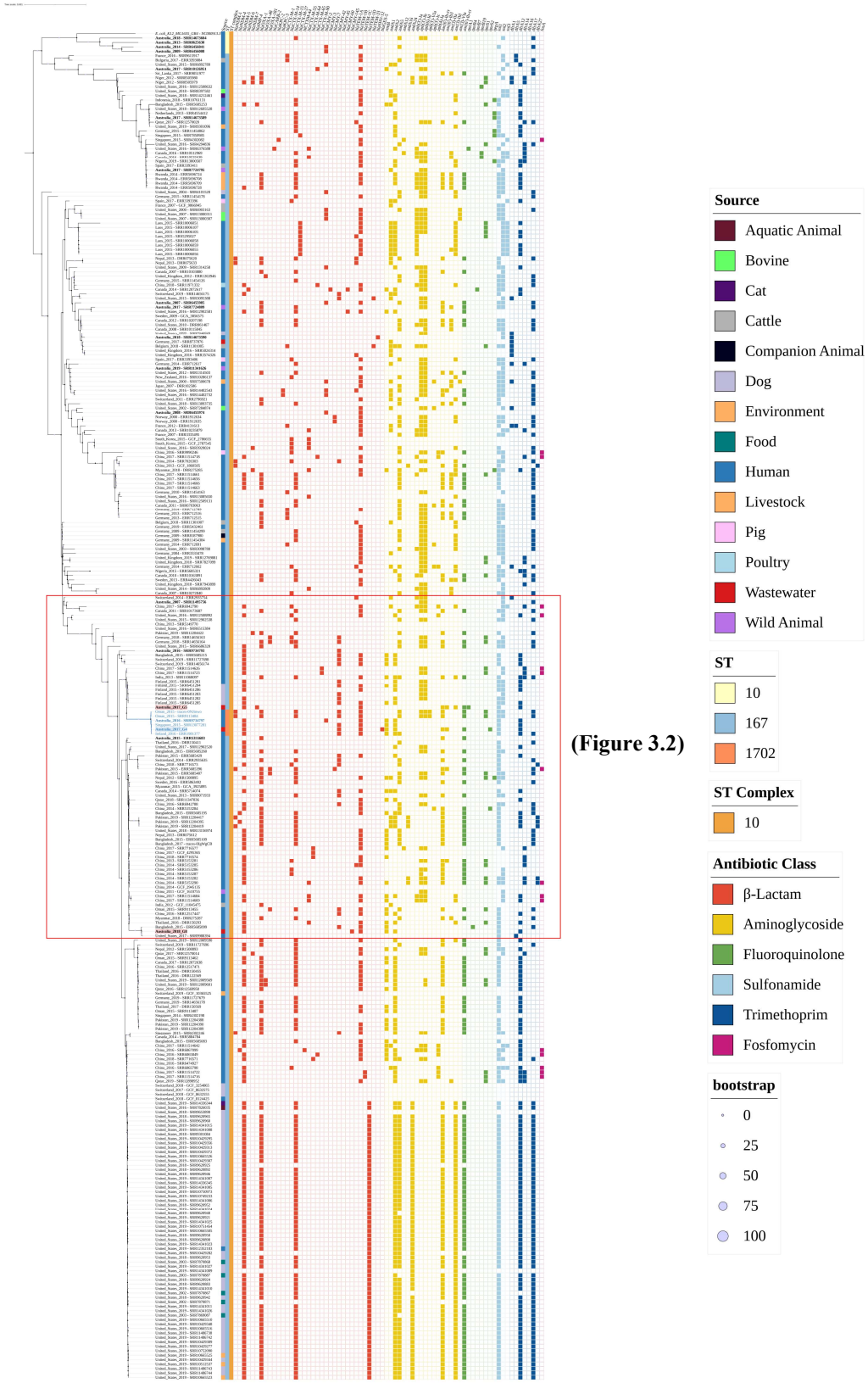


Figure S3.1 Distribution of pan genes, core genes, and soft core genes among the 309 *E. coli* isolates belongs to ST10 clonal complex identified by Roary.



(Figure 3.2)

Figure S3.2 Maximum likelihood phylogeny of 309 ST10, ST167, and ST1702 *E. coli* isolates of the ST10 clonal complex inferred from the alignment of the concatenated core CDS (n = 3,417) determined by Roary and RAxML using the GTR-Gamma model and 100 bootstrap iterations. The tree is midpoint rooted, and the tree nodes are labelled with the origin, year of isolation, and accession number of the isolates. The wastewater isolates are in bold and

highlighted in pink for ST167 and blue for ST1702. The remaining Australian isolates from Enterobase are in bold. The source of the isolates, sequence types (ST), ST complexes, and acquired resistance genes against different clinically important antibiotic classes are annotated on the coloured strips according to the given key. For ease of visualisation and analysis, the tree was pruned at the clade (Figure 3.2) containing wastewater isolates.

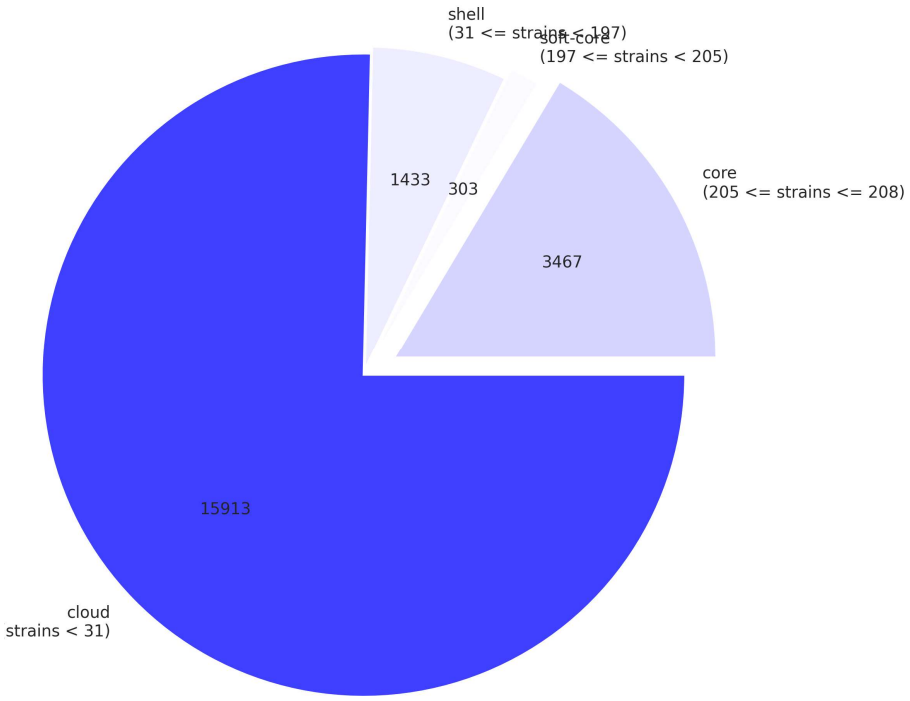
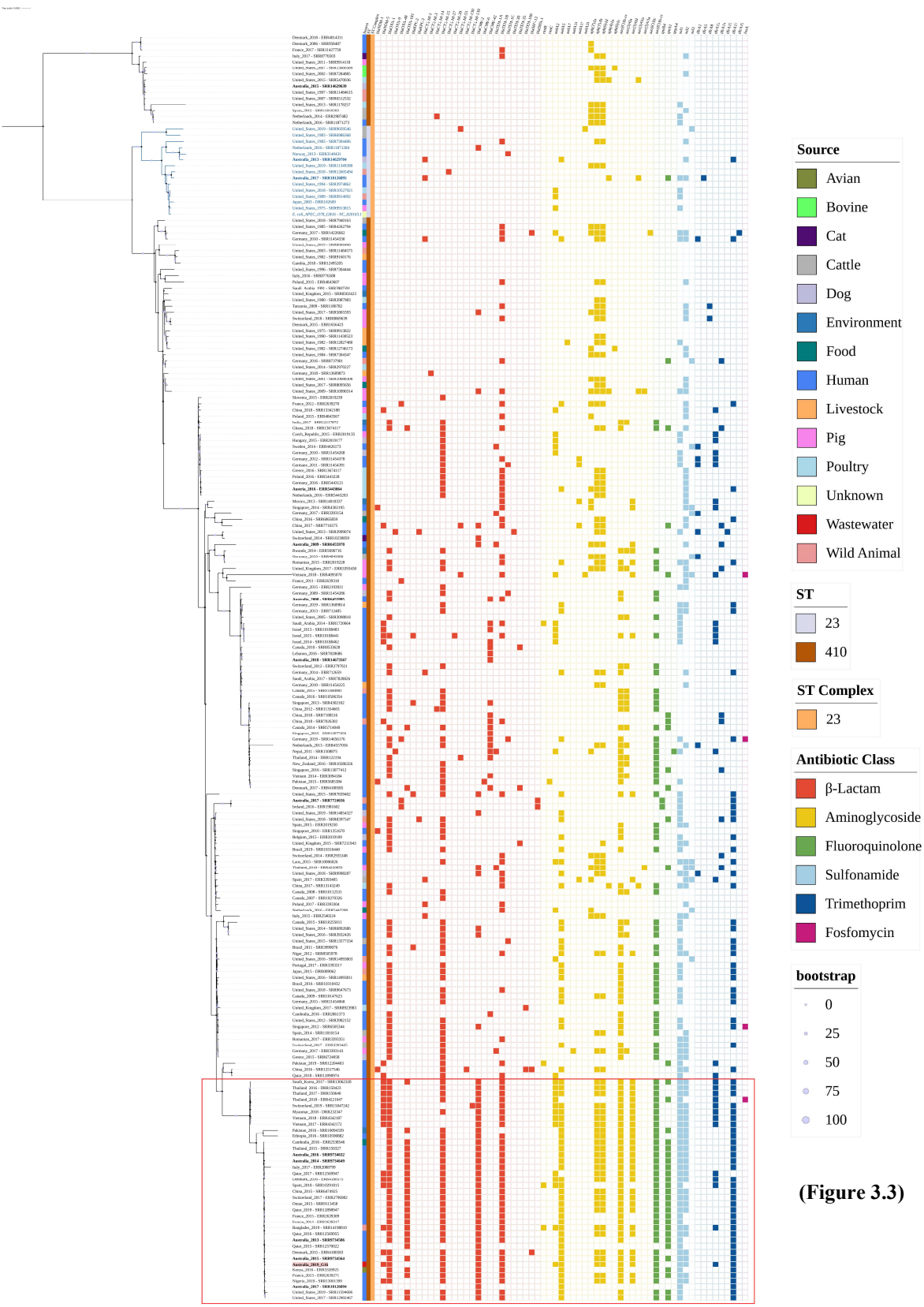


Figure S3.3 The distribution of pan genes, core genes, and soft-core genes among the 208 *E. coli* isolates belonging to the ST23 clonal complex identified by Roary.



(Figure 3.3)

Figure S3.4 Maximum likelihood phylogeny of 208 ST410 and closely related ST23 *E. coli* isolates of the ST23 clonal complex inferred from the alignment of the concatenated core CDS (n = 3,467) determined by Roary and RAxML using the GTR-Gamma model and 100 bootstrap iterations. The tree is midpoint rooted, and the tree nodes are labelled with the origin, year of isolation, and accession number of the isolates. The wastewater isolate belonging to ST410 is

in bold and highlighted in pink. ST23 isolates are highlighted in blue, and the remaining Australian isolates from Enterobase are in bold. The source of the isolates, sequence types (ST), ST complexes, and the acquired resistance genes against different clinically important antibiotic classes are annotated on the coloured strips according to the given key. For ease of visualisation and analysis, the tree was pruned (Figure 3.3) at the clade containing wastewater isolates.

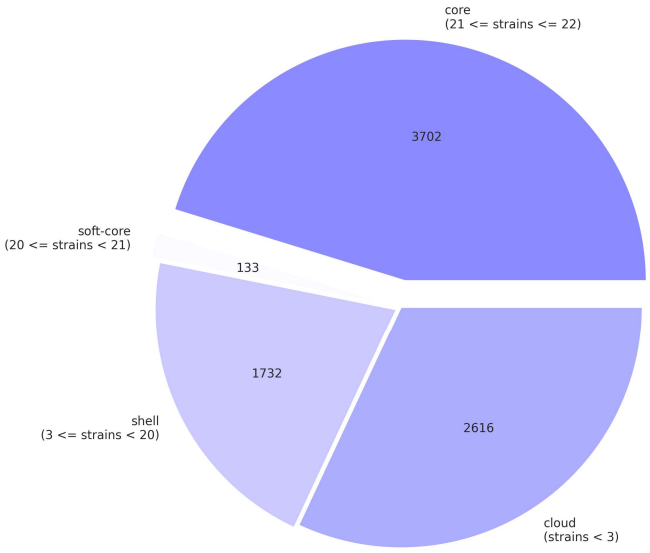


Figure S3.5 The distribution of pan genes, core genes, and soft-core genes among the 22 *E. coli* isolates belonging to ST9586 and closely related ST678 clones identified by Roary.

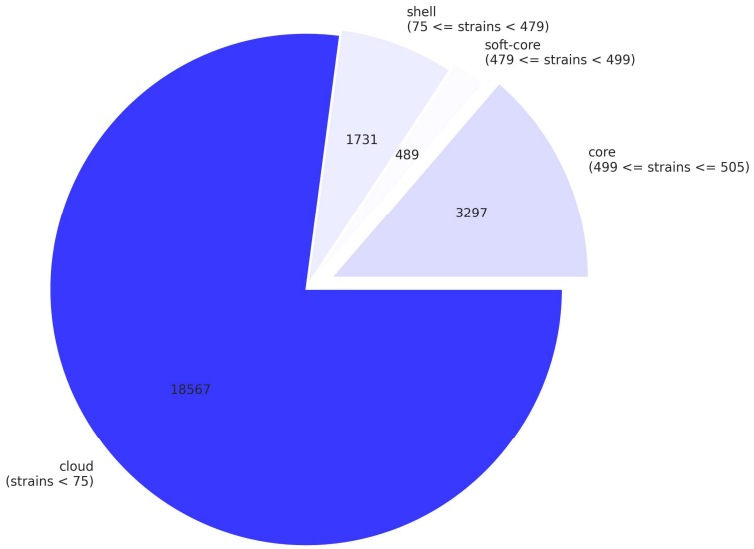
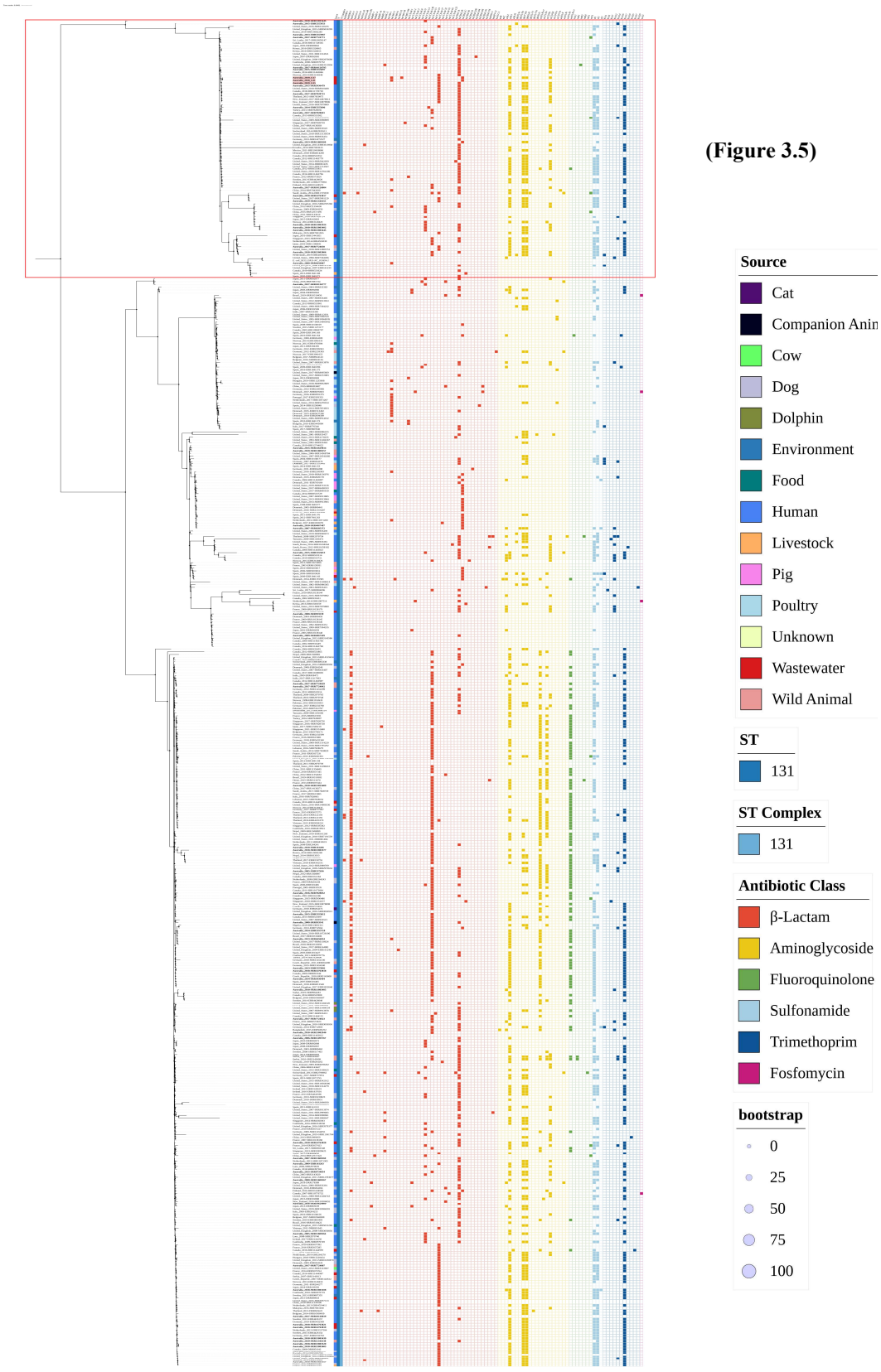


Figure S3.6 The distribution of pan genes, core genes, and soft-core genes among the 505 *E. coli* isolates belonging to ST131 clonal complex identified by Roary.



(Figure 3.5)

Figure S3.7 Maximum likelihood phylogeny of 505 ST131 *E. coli* isolates inferred from the alignment of the concatenated core CDS (n = 3,297) determined by Roary and RAxML using the GTR-Gamma model and 100 bootstrap iterations. The tree is midpoint rooted, and the nodes are labelled with the origin, year of isolation, and accession number of the isolates. Three wastewater isolates belonging to ST131 are in bold and highlighted in pink. The remaining

Australian isolates from Enterobase are in bold. The source of the isolates, sequence types (ST), ST complexes, and the acquired resistance genes against different clinically important antibiotic classes are annotated on the coloured strips according to the given key. For ease of visualisation and analysis, the tree was pruned (Figure 3.5) at the clade containing wastewater isolates.

Appendix C: Chapter 4 Supplementary Information

Supplementary Tables

Table S4.1 Antibiotic susceptibility pattern of the carbapenem-resistant (CR) and extended-spectrum β-lactamases (ESBL)-producing *K. pneumoniae* (KP) (n = 28) and *K. variicola* (KV) (n = 2) species isolated from wastewater-based surveillance in Sydney at six-time points between 2017 and 2019. Isolates selected for whole-genome sequencing are in bold and highlighted in orange for resistant (R), blue for intermediate susceptible (I), and green for susceptible isolates (S).

				Antibiotic Classes																		
				β-lactams							**Penicillins with β-lactamase inhibitors**			**Aminoglycosides**			**Fluoroquinolones**		**Dihydrofolate reductase inhibitors**	**Combinations**	**Nitrofuran derivatives**	**Phosphonic acid derivatives**
Sequenced Genomes	**Month and year of isolation**	**Wastewater catchments**	**Phenotypes**	**Aminopenicillin**	**Cephamycin**	**Cephalosporins**				**Carbapenem**												
				Ampicillin	Cefoxitin	Cefazolin	Ceftazidime	Ceftriaxone	Cefepime	Meropenem	Amoxicillin/clavulanic acid	Piperacillin/tazobactam	Ticarcillin/clavulanic acid	Gentamicin	Tobramycin	Amikacin	Ciprofloxacin	Norfloxacin	Trimethoprim	Trimethoprim-Sulfamethoxazole	Nitrofurantoin	Fosfomycin
G14	**September 2018**	**North Head**	**CR-KP**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**I**	**S**	**R**	**R**	**R**	**R**	**R**	**S**
	June 2019	Malabar	CR-KP	R	R	R	R	R	R	R	R	R	R	R	I	S	R	S	S	S	S	S
	February 2018	Rouse Hill	CR-KP	R	R	S	R	S	S	R	I	S	R	I	S	R	R	S	S	S	I	S
G6	**July 2017**	**Liverpool**	**ESBL-KP**	**R**	**S**	**R**	**R**	**R**	**R**	**S**	**R**	**I**	**R**	**R**	**R**	**S**	**R**	**S**	**R**	**R**	**R**	**S**
G7	**February 2018**	**Quakers Hill**	**ESBL-KP**	**R**	**S**	**R**	**R**	**R**	**R**	**S**	**R**	**I**	**R**	**R**	**R**	**S**	**R**	**S**	**R**	**R**	**R**	**S**
G9	**May 2018**	**Cronulla**	**ESBL-KP**	**R**	**S**	**R**	**R**	**R**	**S**	**S**	**I**	**S**	**S**	**S**	**I**	**S**	**S**	**S**	**R**	**R**	**R**	**S**
	July 2017	Malabar	ESBL-KP	R	S	R	R	R	S	S	R	R	S	S	S	S	S	S	R	S	I	S
	July 2017	Quakers Hill	ESBL-KP	R	S	R	S	R	S	S	I	S	S	R	R	S	I	S	R	R	I	S
	July 2017	Castle Hill	ESBL-KP	R	S	R	I	R	R	S	S	S	S	S	S	S	S	S	R	R	I	S
	July 2017	Shellharbour	ESBL-KP	R	S	R	I	R	S	S	I	S	S	S	R	S	S	S	R	R	I	S

	July 2017	Penrith	ESBL-KP	R	S	R	S	R	S	S	S	S	S	S	S	S	S	S	R	R	S	S
	July 2017	Rouse Hill	ESBL-KP	R	S	R	S	R	S	S	I	S	S	R	I	S	S	S	R	R	I	S
	July 2017	Manly	ESBL-KP	R	S	R	S	R	S	S	S	S	S	S	S	S	S	S	R	R	S	S
	February 2018	Glenfield	ESBL-KP	R	R	S	R	S	S	S	S	S	R	R	R	R	R	S	S	S	I	S
	February 2018	Winmalee	ESBL-KP	R	R	S	R	S	R	I	R	S	R	I	S	R	R	S	S	S	S	S
	February 2018	North Richmond	ESBL-KP	R	R	S	R	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S
	September 2018	Shellharbour	ESBL-KP	R	S	R	R	R	R	S	I	S	I	R	R	S	I	S	R	R	I	S
	September 2018	Glenfield	ESBL-KP	R	S	R	R	R	R	S	S	S	I	S	S	S	S	S	R	R	S	S
	September 2018	Rouse Hill	ESBL-KP	R	S	R	I	R	S	S	I	S	I	S	I	S	S	S	R	S	I	S
	February 2019	West Camden	ESBL-KP	R	I	R	R	R	R	S	I	I	I	S	S	S	S	S	S	S	R	S
	February 2019	West Hornsby	ESBL-KP	R	S	R	S	R	S	S	R	S	I	S	I	S	R	R	R	S	I	S
	February 2019	Shellharbour	ESBL-KP	R	S	R	R	R	R	S	S	S	S	R	I	S	R	S	R	R	I	S
	February 2019	Riverstone	ESBL-KP	R	S	R	R	R	R	S	S	S	S	S	S	S	R	S	R	S	I	S
	February 2019	St Marys	ESBL-KP	R	S	R	R	R	R	S	S	S	S	S	S	S	S	S	R	R	I	S
	February 2019	Brooklyn	ESBL-KP	R	R	R	R	R	R	S	R	S	R	S	S	S	S	S	R	R	I	S
	February 2019	Penrith	ESBL-KP	R	S	R	R	R	R	S	R	I	R	R	I	S	S	S	S	S	S	S
	February 2019	Bondi	ESBL-KP	R	R	R	R	R	R	S	R	R	R	R	R	S	R	S	R	R	I	S
	June 2019	Rouse Hill	ESBL-KP	R	S	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S
G13	**September 2018**	**Liverpool**	**CR-KV**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**I**	**S**	**R**	**I**	**R**	**R**	**R**	**S**
G12	**September 2018**	**Bondi**	**CR-KV**	**R**	**R**	**R**	**R**	**R**	**R**	**I**	**R**	**R**	**R**	**S**	**I**	**S**	**S**	**S**	**S**	**S**	**S**	**S**
Categories of important antibiotics for human medicine (WHO, 2019b)				Critically important (High priority)	Highly important	Highly important	Critically important (Highest priority)	Critically important (Highest priority)	Critically important (Highest priority)	Critically important (High priority)	Critically important (High priority)	Critically important (High priority)	Not available	Critically important (High priority)	Critically important (High priority)	Critically important (High priority)	Critically important (Highest priority)	Critically important (Highest priority)	Highly important	Highly important	Important	Critically important (High priority)

Table S4.2 Publicly available *K. pneumoniae* genomes included in this study.

Country	Year	Accession/ID	Source	Sample	Sequence type	Strain	Contig number	N50 (bp)	Approximate genome size (bp)	Dataset
Italy	2013	NZ_UKNP00000000.1	Human	Wound	ST2791	EuSCAPE_IT059	224	157,213	5,550,581	NCBI_refseq (EuSCAPE)
Japan	2015	SAMD00092905	Wastewater	Influent wastewater	ST2791	JSWP035	228	104,487	6,010,944	Gomi et al., 2018
Colombia	2005	NZ_NCLV00000000.1	Human	Unknown	ST353	COL-Kpn16	57	415,505	5,639,589	NCBI_refseq
Italy	2017	14387	Human	Bronchial	ST353	SPARK_866_C1	42	361,145	5,477,651	Institut Pasteur's BIGSdb-Kp database
Serbia	2013	NZ_UJCN00000000.1	Human	Lower respiratory tract secretion	ST353	EuSCAPE_RS061	142	260,809	5,593,915	NCBI_refseq (EuSCAPE)
Spain	2014	NZ_ULDJ00000000.1	Human	Wound	ST353	EuSCAPE_ES217	453	348,620	5,729,273	NCBI_refseq (EuSCAPE)
Thailand	2016	NZ_UFFQ00000000.1	Human	Unknown	ST353	4300STDY6470469	56	415,065	5,414,813	NCBI_refseq
Turkey	2013	NZ_UJUN00000000.1	Human	Urine	ST353	EuSCAPE_TR303	126	374,416	5,420,632	NCBI_refseq (EuSCAPE)
United Kingdom	2010	NZ_FLGJ00000000.1	Human	Blood	ST353	k2335	45	360,770	5,471,091	NCBI_refseq
Australia	2009	SAMN06112128	Human	Blood	ST13	A0112442	75	304,269	5,412,861	Wyres et al. 2016
Australia	2013	SAMEA3357259	Human	Urine	ST13	INF269	46	562,386	5,461,158	Gorrie et al., 2018
China	2012	NZ_RPFA00000000.1	Human	Blood	ST13	CL2074	86	348,647	5,502,955	NCBI_refseq
China	2014	NZ_QFMO00000000.1	Poultry	Cloaca swabs from commercial farm	ST13	DZ3-14B	499	26,188	5,515,441	NCBI_refseq
China	2015	NZ_QFMP00000000.1	Poultry	Cloaca swabs from commercial farm	ST13	2DZ2F15B	142	64,375	5,460,093	NCBI_refseq
Colombia	2004	NZ_NCLS00000000.1	Human	Unknown	ST13	COL-Kpn13	57	314,902	5,624,672	NCBI_refseq
Colombia	2013	NZ_NCOZ00000000.1	Human	Unknown	ST13	COL-Kpn99	56	342,711	5,480,979	NCBI_refseq
Colombia	2014	NZ_NCPI00000000.1	Human	Unknown	ST13	COL-Kpn108	73	186,507	5,479,163	NCBI_refseq
Cyprus	2014	NZ_UJDM00000000.1	Human	Blood	ST13	EuSCAPE_CY002	277	252,626	5,445,120	NCBI_refseq (EuSCAPE)
Spain	2012	NZ_JABNMQ000000000.1	Human	Urine	ST13	K1620	91	144,823	5,585,428	NCBI_refseq
Spain	2013	NZ_WRWZ00000000.1	Human	Rectal	ST13	ST13-OXA48	91	144,823	5,5854,28	NCBI_refseq
Spain	2014	NZ_ULFA00000000.1	Human	Urine	ST13	EuSCAPE_ES288	54	428,985	5,385,722	NCBI_refseq (EuSCAPE)
France	2018	NZ_VUBI00000000.1	Human	Unknown	ST13	195H2	73	241,578	5,503,623	NCBI_refseq
France	2018	NZ_VUBL00000000.1	Human	Unknown	ST13	195D9	203	127,372	5,596,899	NCBI_refseq
France	2018	NZ_VUBP00000000.1	Human	Unknown	ST13	192J9	56	285,960	5,455,376	NCBI_refseq
France	2018	NZ_VUBT00000000.1	Human	Unknown	ST13	190A4	89	141,740	5,476,051	NCBI_refseq
France	2018	NZ_VUBU00000000.1	Human	Unknown	ST13	189J8	76	186,101	5,448,884	NCBI_refseq
France	2018	NZ_VUBX00000000.1	Human	Unknown	ST13	187H6	59	241,997	5,458,618	NCBI_refseq
France	2018	NZ_VUCQ00000000.1	Human	Unknown	ST13	176G10	67	176,968	5,292,829	NCBI_refseq
United Kingdom	2010	UOAV00000000.1	Human	Hip	ST13	unknown	129	189,911	5,503,728	Ellington et al., 2019
United Kingdom	2011	UOBE00000000.1	Human	Blood	ST13	unknown	110	199,940	5,498,175	Ellington et al., 2019
United Kingdom	2013	NZ_UJHF00000000.1	Human	Urine	ST13	EuSCAPE_UK023	100	479,270	5,406,784	NCBI_refseq (EuSCAPE)
United Kingdom	2014	NZ_UJAP00000000.1	Human	Urine	ST13	EuSCAPE_UK070	98	545,232	5,510,121	NCBI_refseq (EuSCAPE)
Germany	2014	NZ_UIEX00000000.1	Human	Unknown	ST13	ID_54	54	746,313	5,274,318	NCBI_refseq
Germany	2014	NZ_UIFF00000000.1	Human	Unknown	ST13	ID_52	60	746,891	5,274,311	NCBI_refseq
Ireland	2017	6707	Human	Unknown	ST13	ME170770	95	402,798	5,561,770	Institut Pasteur's BIGSdb-Kp database
Ireland	2018	12708	Environment	Seawater	ST13	B18080	90	371,908	5,233,812	Institut Pasteur's BIGSdb-Kp database
Israel	2014	NZ_UKPE00000000.1	Human	Wound	ST13	EuSCAPE_IL061	61	349,083	5,716,627	NCBI_refseq (EuSCAPE)
Israel	2014	NZ_UKPU00000000.1	Human	Urine	ST13	EuSCAPE_IL082	51	348,966	5,432,016	NCBI_refseq (EuSCAPE)
Israel	2017	NZ_SPUC00000000.1	Human	Rectal	ST13	NICU_1_P5	70	141,590	5,253,724	NCBI_refseq

Italy	2017	14379	Human	Urine	ST13	SPARK_858_C1	36	642,263	5,467,141	Institut Pasteur's BIGSdb-Kp database
Italy	2018	13830	Dog	Rectal swab	ST13	SPARK_2647_C2	23	977,699	5,449,965	Institut Pasteur's BIGSdb-Kp database
Italy	2018	12802	Human	Faeces	ST13	SPARK_1024_C1	42	389,260	5,463,500	Institut Pasteur's BIGSdb-Kp database
Italy	2018	12937	Human	Urine	ST13	SPARK_1163_C1	49	378,412	5,469,676	Institut Pasteur's BIGSdb-Kp database
Italy	2018	13059	Human	Urine	ST13	SPARK_1298_C1	49	387,073	5,466,823	Institut Pasteur's BIGSdb-Kp database
Italy	2018	13377	Human	Bronchoalveolar lavage	ST13	SPARK_1774_C1	25	521,214	5,386,973	Institut Pasteur's BIGSdb-Kp database
Italy	2018	13383	Human	Urine	ST13	SPARK_1795_C1	42	518,148	5,466,307	Institut Pasteur's BIGSdb-Kp database
Italy	2018	13520	Human	Urine	ST13	SPARK_2004_C1	25	753,798	5,454,707	Institut Pasteur's BIGSdb-Kp database
Morocco	2017	NZ_FWYR00000000.1	Human	Urine	ST13	KP03M	98	165,526	5,461,352	NCBI_refseq
Nigeria	2015	NZ_JAAASY000000000.1	Human	Urine	ST13	2840	146	167,893	5,607,401	NCBI_refseq
Nigeria	2015	NZ_NFYD00000000.1	Human	Blood	ST13	BH3723	85	168,524	5,450,755	NCBI_refseq
Pakistan	2010	NZ_FXOT00000000.1	Human	Blood	ST13	3189STDY5864760	35	747,017	5,458,676	NCBI_refseq
Pakistan	2010	NZ_FXOU00000000.1	Human	Urine	ST13	3189STDY5864765	37	748,568	5,459,753	NCBI_refseq
Turkey	2013	NZ_UJVE00000000.1	Human	Wound	ST13	EuSCAPE_TR180	63	372,209	5,534,747	NCBI_refseq (EuSCAPE)
United States	2001	NZ_SUNS00000000.1	Human	Unknown	ST13	AS007969	137	112,376	5,604,203	NCBI_refseq
United States	2011	NZ_LOEI00000000.1	Human	Blood	ST13	OC511	234	82,924	5,653,094	NCBI_refseq
United States	2011	NZ_QBCF00000000.1	Human	Urine	ST13	OC743	189	78,146	5,585,918	NCBI_refseq
United States	2014	NZ_CP014123	Human	Urine	ST13	FDAARGOS_156	5	264,538	5,550,639	NCBI_refseq
United States	2017	NZ_RCZZ00000000.1	Human	Patient fluids	ST13	KLPN_37	2	241,999	5,463,832	NCBI_refseq
United States	1999	NZ_SUOH00000000.1	Human	Unknown	ST13	AS004570	133	119232	5,599,387	NCBI_refseq
Australia	2013	SAMEA3357034	Human	Urine	ST268	INF055	57	422,003	5,329,217	Gorrie et al., 2018
Australia	2013	SAMEA3357048	Human	Blood	ST268	INF141	56	407,641	5,329,216	Gorrie et al., 2018
Australia	2013	SAMEA3357114	Human	Urine	ST268	INF095	54	422,003	5,329,217	Gorrie et al., 2018
Belgium	2014	NZ_UJSQ00000000.1	Human	Puncture fluid	ST268	EuSCAPE_BE107	92	275503	5589172	NCBI_refseq (EuSCAPE)
China	2014	NZ_NPHL00000000.1	Human	Blood	ST268	K604	65	340,693	5,669,428	NCBI_refseq
China	2014	NZ_QSOA00000000.1	Human	Faecal	ST268	TM10-8AC	52	350,100	5,511,749	NCBI_refseq
China	2015	NZ_RZKG00000000.1	Human	Sputum	ST268	NJSXKYY21-CR	73	261,856	5,698,846	NCBI_refseq
China	2015	NZ_RZLC00000000.1	Human	Urine	ST268	NJNKYY50-CR	70	261,856	5,736,848	NCBI_refseq
China	2016	NZ_WARE00000000.1	Human	Primary bloodstream infection	ST268	42223	62	317,391	5,529,174	NCBI_refseq
China	2017	NZ_CABWSX000000000.1	Human	Sputum	ST268	ZKP52	158	222,990	5,460,434	NCBI_refseq
China	2017	NZ_CABWTX000000000.1	Human	Liver abscess	ST268	ZKP59	241	306,078	5,450,983	NCBI_refseq
China	2017	NZ_CABWVP000000000.1	Human	Skin and soft tissue fluids	ST268	ZKP143	125	239,390	5,533,288	NCBI_refseq
China	2017	NZ_WAQZ00000000.1	Human	Abdominal infection	ST268	47507	43	318,837	5,408,882	NCBI_refseq
France	2018	WEKU00000000.1	Human	Unknown	ST268	185B2	348	37,638	5,485,306	NCBI_refseq
Italy	2006	CCHC00000000.1	Human	Unknown	ST268	S_52BG	142	96,301	5,494,449	NCBI_refseq
Italy	2007	13368	Human	Urine	ST268	SPARK_176_C1	21	477,657	5,257,683	Institut Pasteur's BIGSdb-Kp database
Italy	2007	14150	Human	Urine	ST268	SPARK_547_C1	45	399,360	5,507,859	Institut Pasteur's BIGSdb-Kp database
Italy	2007	14172	Human	Faeces	ST268	SPARK_59_C1	72	363,414	5,516,495	Institut Pasteur's BIGSdb-Kp database
Italy	2014	NZ_UKVA00000000.1	Human	Urine	ST268	EuSCAPE_IT145	93	295,440	5,467,666	NCBI_refseq (EuSCAPE)
Japan	2013	NZ_BIKW00000000.1	Human	Unknown	ST268	TUM14123	81	329,847	5,540,054	NCBI_refseq
Japan	2014	NZ_BILB00000000.1	Human	Unknown	ST268	TUM14128	95	269,094	5,424,376	NCBI_refseq
Japan	2016	NZ_AP019665.1	Human	Ascites	ST268	TA6363	4	318,671	5,655,563	NCBI_refseq
Malaysia	2013	NZ_JQSE00000000.1	Human	Blood	ST268	349	159	79,370	5,242,964	NCBI_refseq
Russia	2017	NZ_VOOG00000000.1	Human	Feces	ST268	KZN_INI_KINF_60	106	263,911	6,015,114	NCBI_refseq

Spain	2014	NZ_UISN00000000.1	Human	Urine	ST268	EuSCAPE_ES182	541	318,788	5,594,899	NCBI_refseq (EuSCAPE)
Spain	2014	NZ_ULER00000000.1	Human	Lower respiratory tract secretion	ST268	EuSCAPE_ES259	97	260,992	5,429,935	NCBI_refseq (EuSCAPE)
Taiwan	2015	CP041023.1	Human	Sputum	ST268	KP1692	2	370,920	5,573,436	NCBI_refseq
Taiwan	2015	NZ_CP041022.1	Human	Sputum	ST268	KP1677	1	371,084	5,371,084	NCBI_refseq
Thailand	2016	NZ_UFBQ00000000.1	Human	Unknown	ST268	4300STDY6636966	67	318,852	5,655,366	NCBI_refseq
Thailand	2016	NZ_UFES00000000.1	Human	Unknown	ST268	4300STDY6470433	53	368,058	5,352,631	NCBI_refseq
Thailand	2016	NZ_UIRE00000000.1	Human	Unknown	ST268	4300STDY6470394	70	404,970	5,564,507	NCBI_refseq
United Kingdom	2015	NZ_FWIO00000000.1	Human	Faecal	ST268	VRES1176	48	374,222	5,438,082	NCBI_refseq
United Kingdom	2015	NZ_FWIX00000000.1	Human	Faecal	ST268	VRES1171	50	373,873	5,434,546	NCBI_refseq
United Kingdom	2015	NZ_FWIY00000000.1	Human	Faecal	ST268	VRES1170	60	374,010	5,430,017	NCBI_refseq
United Kingdom	2015	NZ_FWJD00000000.1	Human	Faecal	ST268	VRES1175	51	374,250	5,437,702	NCBI_refseq
United Kingdom	2015	NZ_FWJE00000000.1	Human	Faecal	ST268	VRES1179	53	368,807	5,433,791	NCBI_refseq
United Kingdom	2015	NZ_FWJQ00000000.1	Environment	Environmental	ST268	VRES1446	54	368,503	5,436,098	NCBI_refseq
United Kingdom	2015	NZ_FWJT00000000.1	Environment	Environmental	ST268	VRES1462	49	368,700	5,435,665	NCBI_refseq
United Kingdom	2015	NZ_FWJY00000000.1	Environment	Environmental	ST268	VRES1448	51	368,509	5,435,189	NCBI_refseq
United Kingdom	2015	NZ_FWKF00000000.1	Human	Faecal	ST268	VRES1173	51	361,613	5,435,557	NCBI_refseq
United Kingdom	2015	NZ_FWKI00000000.1	Human	Faecal	ST268	VRES1174	50	361,611	5,433,719	NCBI_refseq
United Kingdom	2015	NZ_FWKJ00000000.1	Human	Faecal	ST268	VRES1172	49	473,134	5,432,270	NCBI_refseq
United Kingdom	2015	NZ_FWKN00000000.1	Human	Faecal	ST268	VRES1177	45	368,090	5,434,894	NCBI_refseq
United Kingdom	2015	NZ_FWKR00000000.1	Human	Faecal	ST268	VRES1178	51	362,003	5,435,208	NCBI_refseq
United Kingdom	2015	NZ_FWKT00000000.1	Environment	Environmental	ST268	VRES1458	47	361,627	5,433,655	NCBI_refseq
United Kingdom	2015	NZ_FWML00000000.1	Human	Faecal	ST268	VRCO0340	71	367,790	5,427,472	NCBI_refseq
United Kingdom	2015	NZ_FWMO00000000.1	Human	Faecal	ST268	VRCO0342	57	368,743	5,434,713	NCBI_refseq
United Kingdom	2015	NZ_FWOO00000000.1	Human	Faecal	ST268	VRCO0341	47	368,017	5,432,383	NCBI_refseq
United Kingdom	2015	NZ_FWPD00000000.1	Human	Faecal	ST268	VRCO0339	48	367,701	5,435,098	NCBI_refseq
United Kingdom	2015	NZ_FWPQ00000000.1	Human	Faecal	ST268	VRCO0343	48	367,879	5,435,042	NCBI_refseq
United Kingdom	2018	NZ_CABGUO0000000000.1	Human	Faecal	ST268	4928STDY7071114	74	352,779	5,514,140	NCBI_refseq
United Kingdom	2018	NZ_CABGVG000000000.1	Human	Faecal	ST268	4928STDY7071113	69	352,779	5,512,612	NCBI_refseq
United Kingdom	2018	NZ_CABGVH000000000.1	Human	Faecal	ST268	4928STDY7071116	63	352,908	5,513,735	NCBI_refseq
United Kingdom	2018	NZ_CABGVI000000000.1	Human	Faecal	ST268	4928STDY7071115	67	352,779	5,515,081	NCBI_refseq
United Kingdom	2018	NZ_CABHJH000000000.1	Human	Faecal	ST268	4928STDY7387944	73	372,637	5,514,500	NCBI_refseq
United Kingdom	2018	NZ_CABHJM000000000.1	Human	Faecal	ST268	4928STDY7387945	80	352,848	5,519,059	NCBI_refseq
United Kingdom	2018	NZ_CABHJR000000000.1	Human	Faecal	ST268	4928STDY7071110	64	372,609	5,515,774	NCBI_refseq
United Kingdom	2018	NZ_CABHKB000000000.1	Human	Faecal	ST268	4928STDY7071112	73	352,779	5,515,882	NCBI_refseq
United States	2016	NZ_VKXG00000000.1	Human	Lung	ST268	AS012315	712	94,219	5,489,881	NCBI_refseq
Zambia	2019	17407	Environment	Surfaces	ST268	RL-04	157	146,105	5,538,534	Institut Pasteur's BIGSdb-Kp database
Zambia	2019	17408	Environment	Surfaces	ST268	RL-05	134	189,318	5,547,158	Institut Pasteur's BIGSdb-Kp database
Zambia	2019	17409	Environment	Surfaces	ST268	RL-28	135	218,718	5,546,664	Institut Pasteur's BIGSdb-Kp database
Zambia	2019	17410	Environment	Surfaces	ST268	RL-39	132	189,318	5,545,832	Institut Pasteur's BIGSdb-Kp database
Zambia	2019	17411	Environment	Surfaces	ST268	RL-43	133	245,784	5,546,775	Institut Pasteur's BIGSdb-Kp database
Zambia	2019	17414	Environment	Surfaces	ST268	RL-64	142	202,603	5,546,656	Institut Pasteur's BIGSdb-Kp database
Zambia	2019	17415	Environment	Surfaces	ST268	RL-66	125	220,536	5,547,903	Institut Pasteur's BIGSdb-Kp database

Table S4.3 General features of the assembled *K. pneumoniae* and *K. variicola* genomes.

Genomes	Species	Number of reads after adapter and quality trimming	Number of contigs	N50 (Kbp)	Approximate genome size (Mbp)	G + C content (%)	Number of predicted genes	Number of predicted protein coding sequences (CDS)
G14	*K. pneumoniae*	359,386	224	115.82	5.94	56.77	5,752	5,695
G6	*K. pneumoniae*	667,377	46	348.87	5.59	57.06	5,240	5,179
G7	*K. pneumoniae*	391,995	179	100.53	5.95	56.55	5,719	5,658
G9	*K. pneumoniae*	717,691	50	353.57	5.63	57.18	5,321	5,257
G13	*K. variicola*	472,804	67	279.02	6.05	56.82	5,830	5,763
G12	*K. variicola*	757,793	67	280.48	5.52	57.34	5,259	5,194

Table S4.4 List of contigs co-harbouring plasmid replicons and antibiotic resistance genes in the assembled genome of *Klebsiella* isolates from wastewater.

Isolates	Phenotypes	Contig (size bp)	Plasmid replicon (position in contig)	Resistance gene (position in contig)
G14	CR-KP	NODE_28 (67200)	IncM2 (64617 to 65280)	*bla*$_{\text{TEM-1B}}$ (5944 to 6804)
		NODE_28 (67200)	IncM2 (64617 to 65280)	*aac(3)-IId* (112 to 972)
G12	CR-KV	NODE_44 (9684)	IncQ2 (4873 to 5322)	*qnrS2* (2585 to 3241)

Supplementary Figures

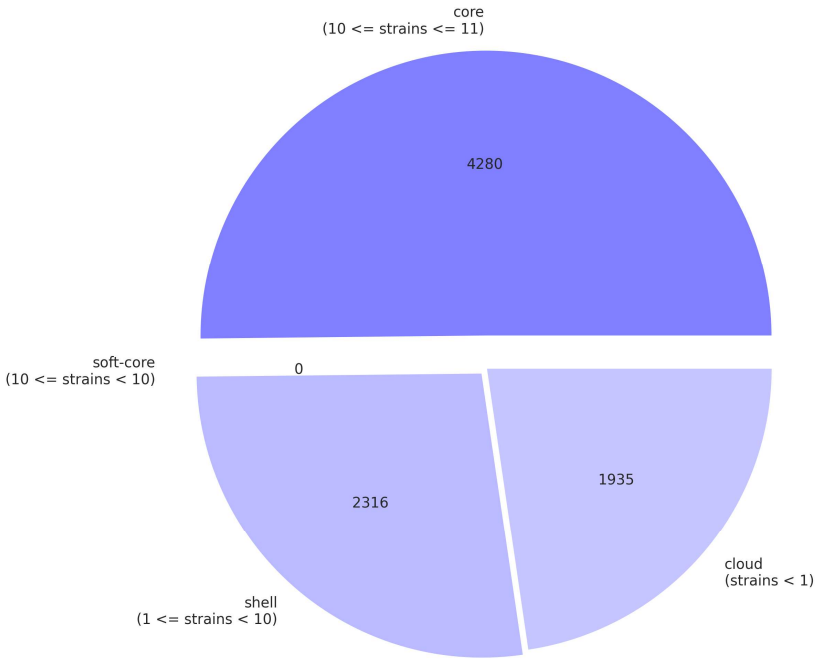


Figure S4.1 Distribution of pan genes, core genes, and soft core genes among the 11 *K. pneumoniae* isolates belongs to ST353 and ST2791 clones identified by Roary.

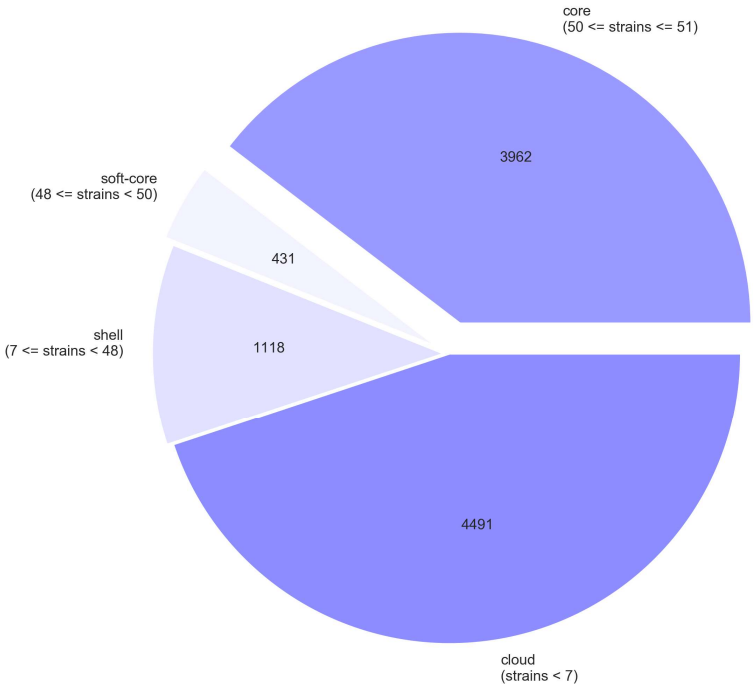


Figure S4.2 The distribution of pan genes, core genes, and soft-core genes among the 51 *K. penumoniae* isolates belonging to the ST13 clone identified by Roary.

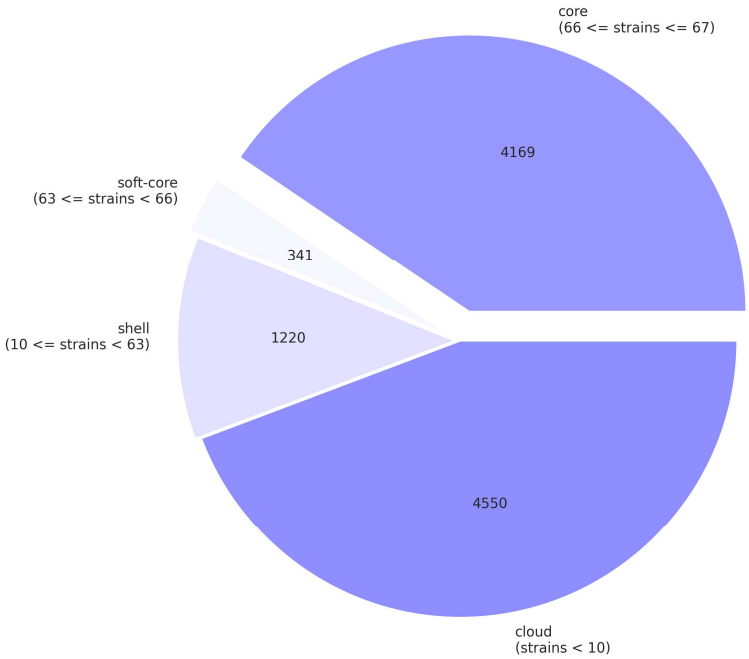


Figure S4.3 The distribution of pan genes, core genes, and soft-core genes among the 67 *K. penumoniae* isolates belonging to the ST268 clone identified by Roary.

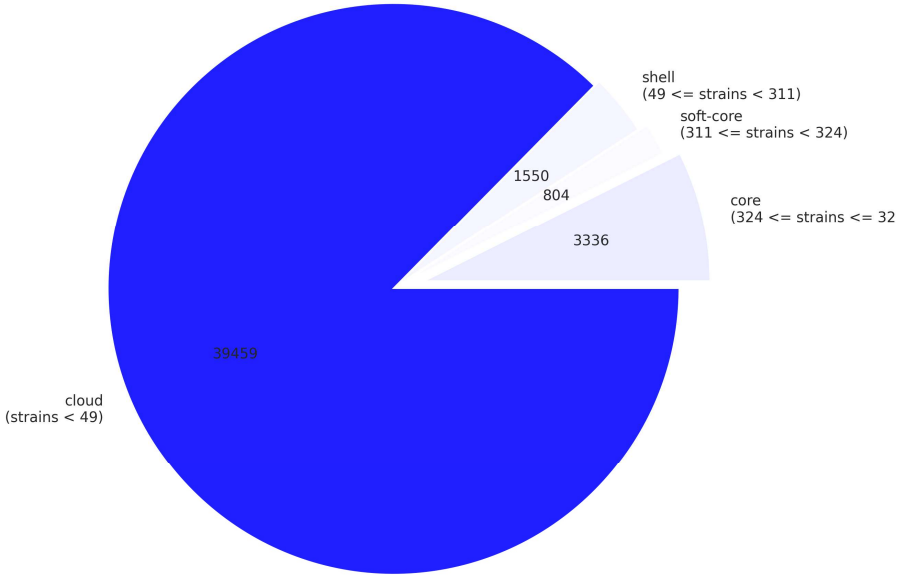


Figure S4.4 The distribution of pan genes, core genes, and soft-core genes among the 328 *K. variicola* isolates belonging to the diveres STs identified by Roary.

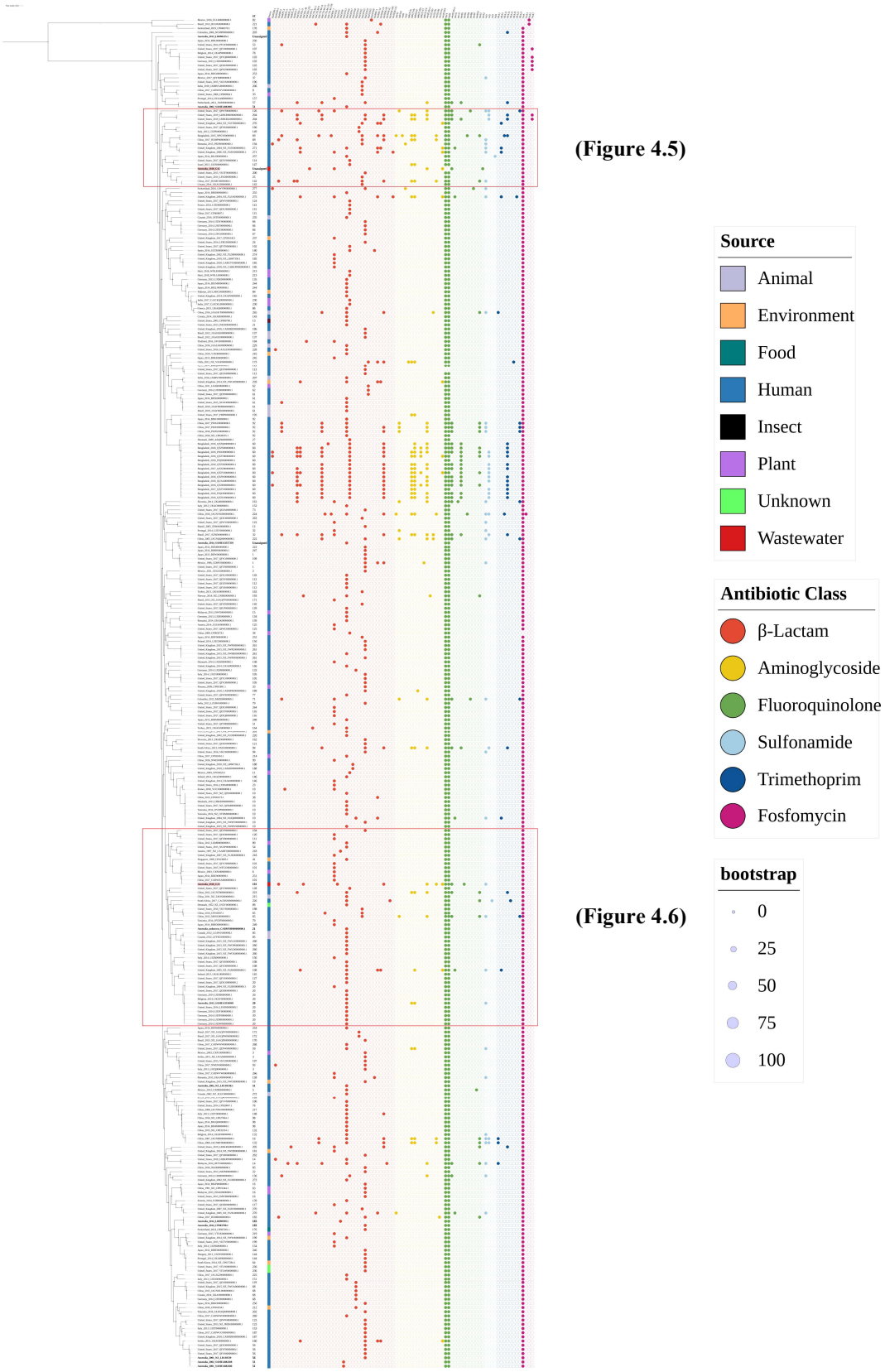


Figure S4.5 Maximum likelihood phylogeny of 328 *K. variicola* isolates belonging to diverse STs inferred from the alignment of the concatenated core CDS (n = 3,336) determined by Roary and RAxML using the GTR-Gamma model and 100 bootstrap iterations. The tree is midpoint rooted, and the tree nodes are labelled with the origin, year of isolation, and accession number of the isolates, followed by the sequence types (ST) of the isolates identified based on the *K.*

variicola MLST scheme (http://mlstkv.insp.mx/). The wastewater isolates are in bold and highlighted in pink, and the remaining Australian isolates are in bold. The source of the isolates and acquired resistance genes against different clinically important antibiotic classes are annotated on the coloured strips according to the given key. For ease of visualisation and analysis, the tree was pruned at the clades containing wastewater isolates G12 (Figure 4.5) and G13 (Figure 4.6) belonging to novel ST and ST101, respectively.

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