Antimicrobial resistance within Irish hospital sanitary ware and bloodstream infections.

Shannon Carter B.Sc. (Hons)

Maynooth University
National University of Ireland Maynooth

HRB
An Bord Taighde Sláinte
Health Research Board

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Supervisor	Head of Department
Prof. Fiona Walsh	Prof. Paul Moynagh

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Declaration of Authorship

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Signed----------

Date----------

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Public Speaking: Poster and Oral presentations

The Microbiology Society Annual Conference, April 2023

Poster presentation: Multi-drug Resistant Pathogens Isolated from the Plumbing Systems of Patient Bathrooms in an Irish Hospital. Emma Rossiter, Shannon Carter, Thuy Thi Do, Marie Gaffney, Cathal Collins, Fiona Walsh.

Maynooth University Annual Research Day, May 2023

Poster presentation: Investigation of hospital sanitary ware as reservoirs of persistent antimicrobial resistant pathogens and links to bloodstream infections: Implications for infection prevention and control. Shannon Carter, Stephen Hughes, Thuy Thi Do, Marie Gaffney, Cathal Collins, Fiona Walsh.

The Microbiology Society Annual Conference and The European Society for Clinical Microbiology and Infectious Diseases, April 2024

Poster presentation: Carbapenem and Colistin resistant Isolates cultured from hospital sanitary ware with a focus on *Stenotrophomonas maltophilia*. Shannon Carter, Michelle O'Reilly, Marie Gaffney, Cathal Collins, Fiona Walsh.

Postgraduate Seminar series, Maynooth, June 2024

Oral presentation: Investigation of hospital sanitary ware as reservoirs of persistent antimicrobial resistant pathogens and links to bloodstream infections: Implications for infection prevention and control. Shannon Carter, Stephen Hughes, Thuy Thi Do, Marie Gaffney, Cathal Collins, Breda O' Brien, Nuala O' Connell, James Powell, Fiona Walsh.

Abbreviations

AMR: Antimicrobial Resistance

AMP: Ampicillin

AMK: Amikacin

BSI: Bloodstream infection

CAZ: Ceftazidime

CIP: Ciprofloxacin

CN: Gentamicin

COL: Colistin

CP: Carbapenemase

CPE: Carbapenemase producing Enterobacterales.

CTX: Cefotaxime

ESBL: Extended-spectrum beta-lactamase

HAIs: Hospital acquired infections.

LEV: Levofloxacin

MIN: Minocycline

MRSA: Methicillin-resistant *Staphylococcus aureus*

VREfm: Vancomycin- resistant *Enterococcus faecium*

TMP/SMX: Trimethoprim/sulfamethoxazole

W: Trimethoprim

Abstract

Antimicrobial resistance (AMR) is a global health crisis with previous reports stating 70% of Hospital Acquired Infections (HAIs) are directly linked to Antimicrobial Resistance (AMR). In Ireland, the rates of AMR associated HAIs increase annually with *Escherichia coli* and *Staphylococcus aureus* the most frequent causative agents. The theory of hospital sanitary ware acting as reservoirs for these AMR pathogens has been previously described throughout Europe.

The first part of the following thesis investigated Irish hospital sanitary ware for the presence of these persistent AMR pathogens with a specific focus on extended-spectrum-beta lactamase (ESBL) and carbapenemase (CP) producing bacteria. Beta-lactams, particularly, carbapenems and third generation cephalosporins, are broad-spectrum antimicrobials used to treat dangerous infections. Pin-pointing the location of AMR pathogens resistant to these life-saving antimicrobials are a top priority within clinical environments. The findings of this section highlight these reservoirs and discuss the microbial populations within.

The second part of the following thesis characterises and compares eighty bloodstream infection (BSI) isolates collected from patients at three Irish hospitals. This section uncovered resistant mechanisms of ESBL and CP Enterobacterales, MRSA, and VREfm. These mechanisms included the presence of $bla_{CTX\text{-}M\text{-}15}$, $bla_{OXA\text{-}48}$, within Enterobacterales, *mecA* genes within MRSA isolates, and the *vanA* operon reported in VREfm isolates. One finding of this study underlines the movement of different plasmid replicon types not only between the bacteria as species but between different species sequence types across all three hospitals. Other findings highlight species relatedness and similarities by using phylogenetic and distancing analysis.

Chapter 1: Introduction

AMR Emergence and Global Action Plans

Since their development antimicrobials such as antibiotics, antifungals, and antivirals have revolutionised modern medicine. These drugs increase life expectancy and function against a wide range of pathogens. However, the actions of these drugs became threatened with the emergence of antimicrobial resistance (AMR) (Toner *et al.*, 2015) (Waksman, Reilly and Schatz, 1945). Antimicrobial resistance occurs when pathogens utilise mechanisms preventing these drugs from functioning. Many resistance mechanisms are due to resistance genes which can be encoded intrinsically on chromosomes or acquired via mobile genetic elements (MGEs) like plasmids, transposons, integrons, or chromosomal complexes. These MGEs are responsible for the increase in AMR dissemination world-wide and across species. Consequently, mobile genetic elements have led to an increased abundance of multidrug resistant (MDR) pathogens.

AMR has become a global burden today and is predicted to be the cause of 10 million deaths by 2050 (O'Neil.,2016). Though this has been disputed by some (Korotetskiy *et al.*, 2022) (de Kraker, Stewardson and Harbarth, 2016), the threat of AMR is not being overlooked. In 2019, it was estimated that 1.27 million deaths were attributed to AMR world-wide (Murray *et al.*, 2022). An Irish study published in 2021(Health Information and Quality Authority, 2021) estimated that €12m was spent to treat patients with resistance-associated infections.

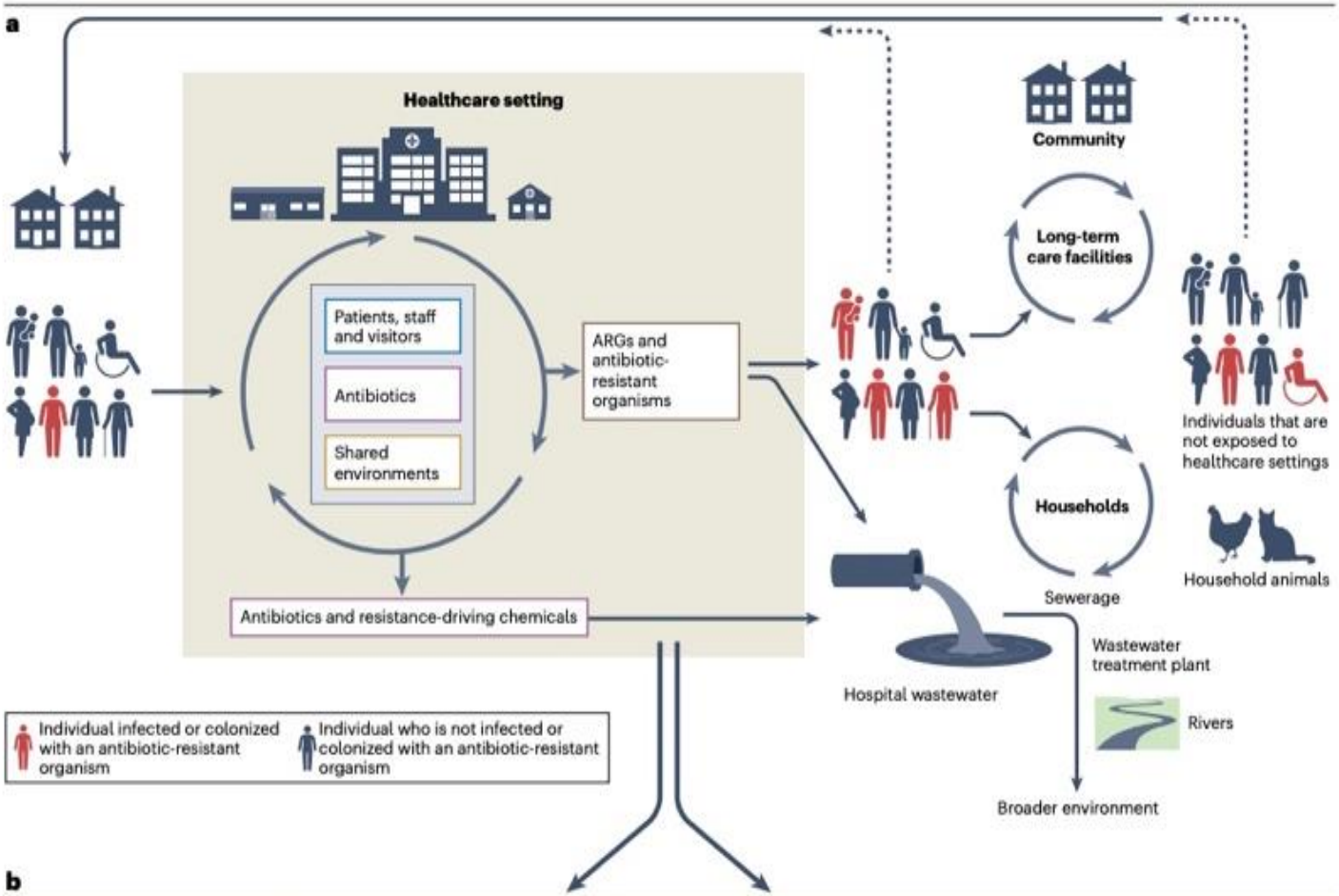
The World Health Organisation (WHO) published a global national action plan to tackle AMR in 2015 and paved the way for country-specific action plans. Following on in 2017, Ireland developed its first National Action Plan on Antimicrobial Resistance (Department of Health, 2017). Further known as iNAP1 the plan was published by both the Departments of Health (DOH) and Agriculture, Food, and the Marine (DAFM) and successfully led to development of Ireland's second One Health National Action Plan against AMR (iNAP2) in 2021 (Government of Ireland, 2021). iNAP2 was put in place from 2021 until 2025 with some of the main objectives being education on AMR, the reduction

of infections linked to this phenomenon, optimising antimicrobial drug usage and prescriptions, and promoting more research in the field (Government of Ireland, 2021).

AMR in Hospital Environments.

According to the European Centre for Disease Prevention and Control (ECDC) it is estimated that 70% of hospital acquired infections (HAIs), also known as nosocomial infections, are directly linked to AMR (Merk et al.,2022). ECDC published a point prevalence survey (PPS) regarding HAIs and antimicrobial use in European acute care hospitals from 2022-2023. Over 22,000 HAIs were reported and over 13,000 of those HAIs involved AMR pathogens. Of those microbes, the Gram-negative, *Escherichia coli* and *Klebsiella* spps, made up 12.7% and 11.7% of the pathogens reported, respectively. Other microorganisms of importance included: *Staphylococcus aureus* (9.0%), *Citrobacter* spps (8.0%), and *Stenotrophomonas maltophilia* (8.0%).

The hospital environment can act as a potential reservoir for AMR. Some of the main driving forces of AMR within hospital settings include high levels of antimicrobial usage, human-human contact, contamination of dry and moist surfaces, and microbe molecular survival processes (Figure 1).


a
Healthcare setting
Patients, staff and visitors
Antibiotics
Shared environments
ARGs and antibiotic-resistant organisms
Antibiotics and resistance-driving chemicals
Community
Long-term care facilities
Households
Individuals that are not exposed to healthcare settings
Household animals
Sewerage
Wastewater treatment plant
Hospital wastewater
Rivers
Broader environment
Individual infected or colonized with an antibiotic-resistant organism
Individual who is not infected or colonized with an antibiotic-resistant organism

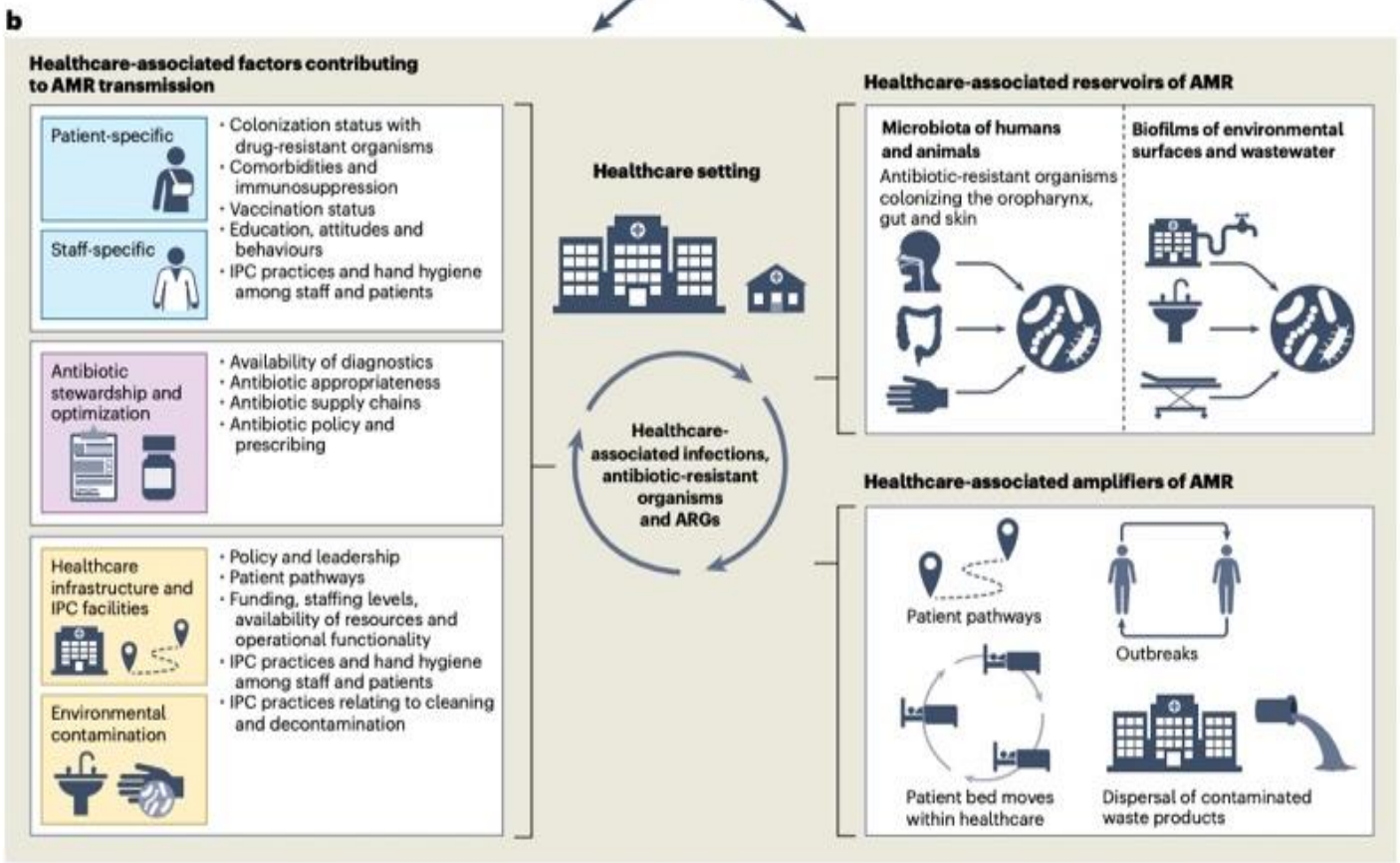

b
Healthcare-associated factors contributing to AMR transmission
Patient-specific
Colonization status with drug-resistant organisms
Comorbidities and immunosuppression
Vaccination status
Staff-specific
Education, attitudes and behaviours
IPC practices and hand hygiene among staff and patients
Antibiotic stewardship and optimization
Availability of diagnostics
Antibiotic appropriateness
Antibiotic supply chains
Antibiotic policy and prescribing
Healthcare infrastructure and IPC facilities
Policy and leadership
Patient pathways
Funding, staffing levels, availability of resources and operational functionality
IPC practices and hand hygiene among staff and patients
Environmental contamination
IPC practices relating to cleaning and decontamination
Healthcare setting
Healthcare-associated infections, antibiotic-resistant organisms and ARGs
Healthcare-associated reservoirs of AMR
Microbiota of humans and animals
Antibiotic-resistant organisms colonizing the oropharynx, gut and skin
Biofilms of environmental surfaces and wastewater
Healthcare-associated amplifiers of AMR
Patient pathways
Outbreaks
Patient bed moves within healthcare
Dispersal of contaminated waste products

Figure 1.1: AMR within the hospital environment (Cocker *et al.*, 2024) ARGs: Antimicrobial Resistance Genes, IPC: Infection, Prevention, and Control.

High levels of antimicrobial use can directly (selection) or indirectly (co-selection) drive AMR. In the presence of increased levels of fluroquinolone antimicrobials, bacterial strains may develop mutations within the *parE* and *parC* genes – causing fluroquinolone resistance - strains with developed resistance will proliferate, producing daughter strains with the same resistance mechanisms (Baquero *et al.*, 2021), thus selecting for fluoroquinolone resistant bacteria. Consequences of selection within hospitals includes colonisation of both the patient and patient environment and there may also be cross colonisation of subsequent patients and hospital staff which results in increasing transmission rates throughout the hospital.

Co-selection results in a pathogenic strain becoming resistant to multiple antimicrobials via different resistance mechanisms typically encoded by acquired genes (Herrick *et al.*, 2014). These acquired genes are typically found on mobile genetic elements like plasmids or integrons. These can then disseminate throughout the hospital and move out to community environments.

Most AMR pathogens can survive within moist environments such as sanitary ware drains, sinks, toilets and waste buckets (Neidhöfer *et al.*, 2023; Valzano *et al.*, 2024), or on hospital inanimate surfaces such as bed posts, countertops, glass, fabrics, and paper (Jabłońska-Trypuć *et al.*, 2022) or adhere to medicinal equipment like pace-makers, dental implants, mechanical heart valves, catheters, or prosthetics (Assefa and Amare, 2022). Antimicrobial resistant pathogens, like most microbes, may form biofilms which function as a process of survival, providing both protection and nutrients. Biofilms comprise an extracellular polymeric matrix that contains compounds and molecules needed for survival. (Johnson *et al.*, 2010; Dahbi *et al.*, 2014; Zhong *et al.*, 2015; De Toro *et al.*, 2017; Welker *et al.*, 2020; Kurittu *et al.*, 2022). Biofilms can be “hydrated” meaning they grow within moist/aquatic environments or “Dry”, meaning they can adhere to inanimate surfaces or medicinal equipment. Due to a range of factors, biofilms are notoriously difficult to eradicate, this becomes especially dangerous with the presence

of AMR pathogens, as antimicrobials or biocides are prevented from reaching these organisms (Sharma, Misba and Khan, 2019). Microbes within the biofilms can further pass on genetic material – such as resistance plasmids- via horizontal gene transfer, which can increase the pathogen population within.

Previous studies have highlighted the presence of AMR pathogens within hospital sanitary ware, typically in shower and sink drains. Neidhöfer *et al* reported carbapenemase producing (*bla*$_{NDM}$, *bla*$_{VIM}$ and *bla*$_{OXA-48}$ genes) *Klebsiella* spp. and *Pseudomonas* spp. isolates within these drains. While in a 2024 study, Valzano *et al*, reported the presence of carbapenemase producing bacteria including, *Citrobacter freundii*, within hospital toilets.

Other studies have shown hospital wastewater as a major source of AMR transmission from hospital settings to the environment or community (Cahill *et al.*, 2019; Perry *et al.*, 2021; Kelly *et al.*, 2023; Siri *et al.*, 2024). In 2019, Cahill *et al*, discovered carbapenemase producing Enterobacterales within the wastewater from hospital effluent and post hospital wastewater while in 2023 Kelly *et al*, concluded isolates collected from hospital wastewater pipes had high genomic similarity to clinically collected isolates, with some genomes identical.

ESKAPE Pathogens and Bloodstream Infection Isolates

Bloodstream infections are caused by a variety of microbes, but a high percentage are caused by the ESKAPE pathogens (Marturano and Lowery, 2019; Gupta *et al.*, 2024). ESKAPE pathogens – *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae, Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp – are superbugs classified as critical or high priority for the development of new antimicrobials by the World Health Organisation (WHO) (Miller and Arias, 2024). The priority pathogens are ESBL producing or carbapenem resistant Gram-negative pathogens, and VRE and methicillin and vancomycin resistant or intermediate *S. aureus*. These pathogens can acquire AMR genes such as the ESBL *bla*$_{CTX-M-15}$ gene (Savin et al.,

2020), carbapenemase *bla*$_{OXA-48}$ gene (O'Connell *et al.*, 2022), or the methicillin resistant *mecA* gene (Gajdács, 2019), allowing for their survival against these antibiotics. Some even acquire gene clusters like the vancomycin resistant, *vanA* operon (Hill *et al.*, 2010; Ahmed and Baptiste, 2018; Leigh *et al.*, 2022).

This study aims to bridge a critical gap between previous research on AMR and the current understanding of hospital sanitary ware reservoirs in Ireland. Data on AMR bacterial pathogenic reservoirs within Irish hospital sanitary ware is limited. Prior studies have predominately focused on either sanitary ware or clinical isolates. Our study aims to investigate both, utilizing molecular and computational methods to evaluate resistant mechanisms and explore potential links between sanitary ware isolates and clinical isolates (BSI isolates).

Thesis Aims:

1. Investigating an Irish hospital's sanitary ware for the presence of antimicrobial resistance (AMR) reservoirs across three timepoints. This investigation aims to gather an understanding of the AMR pathogen population within this sanitary ware.
2. The second aim of this study was to compare several bloodstream isolates collected from patients by three separate Irish hospitals (including the one used for the sanitary ware collection). Whole genome sequencing will be utilised to investigate shared resistance mechanisms, genome relatedness, and mutations.

The rationale behind this study was to provide an understanding of the AMR pathogen populations present in the Irish hospital sanitary ware to the infection prevention and control team within the hospital.

Chapter 2: Investigating AMR in Irish Hospital Sanitary Ware

INTRODUCTION

Antimicrobial Resistance in Hospital Sanitary Ware

The continuing threat of AMR is a global public health concern. Hospitals with increased levels of antimicrobial usage and high patient populations drive AMR dissemination (Struelens, 1998; Nardulli *et al.*, 2022). Antimicrobial resistant pathogens like many different bacterial species thrive in moist areas and several studies have highlighted their presence in hospital sanitary ware (Voigt *et al.*, 2019; Volling *et al.*, 2021; Diorio-Toth *et al.*, 2023; Neidhöfer *et al.*, 2023; Valzano *et al.*, 2024).

Antimicrobial resistance can occur within bacterial populations due to the acquisition of mobile genetic elements (MGEs) – conjugative plasmids, transposons, insertions –by horizontal gene transfer. These MGEs contain virulence and/or metal or antimicrobial resistant genes which increase AMR dissemination. Resistance genes prevent the antimicrobial function while virulence factors contribute to the bacterial pathogenicity. Adhesin virulence factors, such as *fim*-type genes in *E. coli*, contribute to the formation of biofilms. Biofilms are thick extracellular matrices which provide AMR pathogens with nutrients and protection. Previous studies have noted the importance of biofilms within shower and sink drains (Soto-Giron *et al.*, 2016; Proctor *et al.*, 2018; Bourdin *et al.*, 2024; Hayward *et al.*, 2024).

Hospital Associated AMR Pathogens: Carbapenemase and Extended-Spectrum- Beta-Lactamase Producing Enterobacterales and Carbapenem-resistant *Stenotrophomonas maltophilia*.

Enterobacterales are an order of Gram-negative bacteria that include species such as*, E. coli, Citrobacter freundii,* and *Enterobacter* (Janda and Abbott, 2021). Pathogenic lineages of these bacteria are frequently acquired in hospitals as infections. Their importance is even more notable due to their increasing ability to acquire AMR genes and most recently genes conferring resistance to last line of defence antimicrobials, such as the carbapenem and third generation cephalosporin antimicrobials. Cephalosporins

function against bacteria like their penicillin predecessors by targeting cell wall synthesis and inhibiting peptidoglycan integration (Arumugham, Gujarathi and Cascella, 2025). There are currently five generations of cephalosporins which have evolved to expand their efficacy against AMR pathogens. The first to second generation are typically used for those allergic to penicillin while the third generation is utilized against dangerous invasive infections (Cunha, 1992; Araten *et al.*, 2024; Arumugham, Gujarathi and Cascella, 2025). The fourth and fifth generations are typically reserved for Gram (+) infections (Garau *et al.*, 1997; Giamarellou, 1999; Duplessis and Crum-Cianflone, 2011; Bavaro *et al.*, 2024). The chemical structure of the 1st, 2nd and 3rd gen cephalosporins protect these bactericidal agents against penicillinases, though not from ESBL producing bacteria). The evolution of plasmid mediated bacterial ESBLs inhibits the function of first to third generation cephalosporins (Paterson and Bonomo, 2005; Rawat and Nair, 2010). These enzymes evolved due to mutations within beta-lactamases TEM and SHV type genes, $bla_{TEM\text{-}1}$, $bla_{TEM\text{-}2}$, and $bla_{SHV\text{-}1}$ (Chaudhary and Aggarwal, 2004). The enzymes are now very diverse and have disseminated across the globe, with various families such as CTX, TEM, SHV, AmpC, PER and VEB types reported (Ghenea *et al.*, 2022; Rastuti, Budayanti and Dwija, 2023). The CTX- type ESBLs, particularly $bla_{CTX\text{-}M\text{-}15}$, are the most frequently reported type within Irish studies (Morris *et al.*, 2003, 2009; Ludden *et al.*, 2015, 2020). Extended-spectrum beta-lactamase enzymes do not function against carbapenem antimicrobials.

Carbapenems are broad-spectrum β-lactam antimicrobials that are usually reserved as the last line of defence against multi-drug AMR pathogens (Papp-Wallace *et al.*, 2011; Humphreys *et al.*, 2022). Carbapenem resistant pathogens produce enzymes known as carbapenemase enzymes which provide protection against these antimicrobials. There are several types of carbapenemase enzymes from three main classes: class A, class B, and class D. Class A carbapenemase include bla_{GES}, bla_{KPC}, bla_{SME} and are serine carbapenemases while those on class B are also metallo-carbapenemases they include enzymes bla_{NDM}, $bla_{IMP,}$ bla_{VIM}. Enzymes from class D contain serine within their active site and include oxacillinase-type genes like $bla_{OXA\text{-}48}$. Carbapenemase producing Enterobacterales (CPE) infections were first documented in Ireland in 2009 (Cotter *et al.*, 2009), and within six years they were announced as a National Public Health Emergency

(Vellinga *et al.*, 2021). CPE infections are notoriously difficult to treat (Nordmann, Dortet and Poirel, 2012; Taggar *et al.*, 2020; Armstrong, Fenn and Hardie, 2021; Aurilio *et al.*, 2022).

In addition to CPE infections, there are rising numbers of carbapenem intrinsically resistant *Stenotrophomonas maltophilia* documented in hospital environments (Gideskog, Welander and Melhus, 2020; Banar *et al.*, 2023; Cristina *et al.*, 2024). *Stenotrophomonas maltophilia* is a Gram-negative bacterium from the order Xanthomonadales. *Stenotrophomonas maltophilia* was originally considered a low virulence pathogen but has emerged as a pathogen in immunocompromised hosts, and those with cystic fibrosis (Milne and Gould, 2012; Gallagher *et al.*, 2019; Amin, Jahnke and Waters, 2020). In addition to cystic fibrosis, *S. maltophilia* has been associated with diseases such as pneumoniae (Velázquez-Acosta *et al.*, 2018; Campanella *et al.*, 2023; Kızılırmak and Havlucu, 2023; Raad *et al.*, 2023) , endocarditis (Khan and Mehta, 2002; Subhani *et al.*, 2016), meningitis (Platsouka *et al.*, 2002; Yemisen *et al.*, 2008; Khanum, Ilyas and Ali, 2020; Manuel *et al.*, 2021), and sepsis (Papadakis *et al.*, 1995; Verma, Patnaik and Suryawanshi, 2024). It is intrinsically resistance to carbapenems (Howe, 1997; Fihman *et al.*, 2012; Rhoads, 2021), and difficult to diagnose (Aysert-Yıldız *et al.*, 2022), which has resulted in a lack of standardised breakpoints for various antimicrobials (Maraolo *et al.*, 2023; Sarzynski *et al.*, 2023). These factors make *S. maltophilia* infections challenging to treat.

Colistin.

Colistin or Polymyxin E, is used as an antimicrobial treatment when all others fail i.e., a final defence against multi-drug CPE infections in most hospital environments. Although not as prominent as CPE, colistin-resistant pathogens have been noted within several hospital environments (Marchaim *et al.*, 2011; Boo *et al.*, 2013; Osei Sekyere, 2019). One study reported colistin-resistant *Citrobacter freundii* within toilet plumbing systems of three wards (Valzano *et al.*, 2024).

Aims of the study.

As most bacteria thrive in moist environments and previous studies have highlighted the presence of CPE and ESBL-producing pathogen reservoirs within sanitary ware, the aim of this study was to investigate Irish hospital sanitary ware for the presence of these CPE and ESBL- producing pathogens, identify the species of the isolated pathogens, profile their antimicrobial susceptibility, utilize whole genome sequencing to characterize genomic similarity between the isolates, and investigate isolate resistance mechanisms, such as the presence of antimicrobial, biocide, or metal resistance genes. We can then investigate the movement of these genes within bacteria at different timepoints or locations and determine if the genes are present on the chromosome or plasmids of the bacteria. This data will then be provided to the infection, prevention, and control teams within the hospital.

Materials and Methods

Sanitary Ware Isolate Collection

For this study we collaborated with an Irish hospital (Hospital 2) to collect e-swabs from the sanitary ware within three rooms (Room A, Room B, Room C. Shower, sink and toilet surfaces/drains were swabbed once a week over three weeks: from March 30th 2021 to April 13th 2021. Swabs were collected by our collaborators within Hospital 2 and then sent via same-day delivery methods to our laboratory for further analysis.

Isolate Enrichment and Selecting for carbapenemase and extended-spectrum beta-lactamase producing Enterobacterales.

The following work was completed by Dr. Thuy Thi Do

To select for Gram-negative bacteria swabs were enriched in 5 mls of Enterobacteria Enrichment (EE) Broth Mossel and incubated overnight at 37^{O}C in a shaking incubator. Positive cultures (100 µl) were then added to Eosin methylene blue (EMB) and Simmons citrate 1% myo-inositol selective agar plates with added imipenem (8 mg/ml) or cefotaxime (4 mg/ml) and incubated for 24hrs at 37 ^{O}C. Imipenem and cefotaxime concentrations were determined in accordance with the EUCAST breakpoints. *Escherichia coli* ATCC 25922 (susceptible) strains were added to imipenem/cefotaxime agar plates as the antimicrobial quality control. For agar quality control, 100 µl of the positive culture was added to agar plates without antimicrobials. Both controls were incubated with the positive cultures for 24 hours at 37^{O}C. For the following sections each agar plate has been prepped with cefotaxime or imipenem antimicrobials. After 24 hours agar plates were removed from the incubator and any plates with growth underwent serial dilutions at a 1/10 dilution factor in phosphate-buffered saline (PBS). 100 µl was taken from bacterial concentrations of 10^{-3}, 10^{-4}, 10^{-5}, and added to three agar plates, now at the concentrations 10^{-4}, 10^{-5}, and 10^{-6}, these plates were incubated overnight at 37^{O}C. After 24 hours the plates were analyzed and colonies were picked from 10^{-6} dilutions, sub- cultured onto one plate spilt into three sections and incubated overnight at 37^{O}C. The following day, sub-cultured plates were analyzed, and colonies were picked from each section of growth (1 colony per sections 1, 2 and 3) and placed in antimicrobial selected nutrient broth overnight at 37^{o}C in a shaking incubator. After 13-18 hours the positive cultures were examined and prepped within 4% glycerol for storage in -80 cyrofreezers.

Antimicrobial Susceptibility Testing

Kirby-Bauer AST disc diffusions with *C. freundii*, *Enterobacter*, and *E,coli* were performed by Dr. Thuy Thi Do.

Colistin tests and *S. maltophilia* ASTs were performed by Shannon Carter.

Antimicrobial Susceptibility testing (ASTs) was performed using the Kirby-Bauer disc diffusion method (Biemer, J.J., 1973). Enterobacterial isolates (n = 69) were tested for susceptibility to imipenem, ertapenem, meropenem, cefotaxime, ceftazidime, ciprofloxacin, gentamicin, amikacin, and trimethoprim using antimicrobial discs with concentrations in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) 2023/2024 guidelines. These isolates were tested against colistin using the micro-broth dilution method and EUCAST breakpoints. *S. maltophilia* isolates (n = 130) were tested for susceptibility to trimethoprim/sulfamethoxazole, minocycline, and levofloxacin in accordance with breakpoints from the Clinical and Laboratory Standards Institute (CLSI) 2023/2024 guidelines. *E. coli* ATCC 25922 strains were used as quality control strains.

All the work to follow was completed by Shannon Carter.

16S rRNA Microbial DNA Sequencing and Whole Genome Sequencing preparation.

Isolates resistant to imipenem (n =130) and isolates resistant to cefotaxime (n =69) underwent 16S rRNA sequencing to estimate their species identification. 16S rRNA genes within the isolates were amplified using the polymerase chain reactions (PCR) method and universal V3-V4 forward (5' TCG TCG GCA GCG TCA GAT GTG TAT AA 3') and V3-V4 reverse (5' GTC TCG TGG GCT CGG AGA TGT GTA TAA 3') primers for our cefotaxime resistant isolates and the universal 27 forward (5' AGAGTTTGATCCTGGCTCAG-3') and 1492 reverse (5'-TACGGYTACCTTGTTACGACTT-3') primers for our imipenem resistant isolates. 16S rRNA PCR products were evaluated using gel electrophoresis in a 1% agarose gel (Duchefa Biochemie, The Netherlands). The PCR products were then prepared in accordance with the Eurofin Genomics guidelines and sent to Eurofin Genomics (Eurofin Genomics, Germany GMBH, Anzinger St 7a, 85560 Ebersberg, Germany) for sequencing.

Some cefotaxime-resistant/ carbapenem-resistant isolates (n=11) underwent whole genome sequencing. These isolates were selected due to their resistance to ertapenem and cephalosporin antimicrobials. Microbial DNA was extracted from our isolates using the Macherey-Nagel Nucleospin DNA isolation kit (Macherey-Nagel, CmbH Co.KG, Germany) according to the instructions provided. DNA concentrations were evaluated using a NanoDrop ™ spectrophotometer. Degradation quality checks were performed using gel electrophoresis in a 1% agarose gel (Duchefa Biochemie, The Netherlands). The extracted DNA was sent to Novogene Sequencing (Novogene (UK) Company Limited, Cambridge, UK) for short-read whole genome sequencing. The library preparation was performed on a Novseq X plus series illumina platform.

Isolate identification via NCBI BLAST

To investigate the identity of our isolates their sequenced 16S rRNA gene fasta files were inputted into the National Centre for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) and run against the 16S rRNA database. The species that matched our alignment with near complete coverage, percentage identity over 97%, and had a low E-value score was our estimated species identity.

Genome annotation and assembly

The cefotaxime/carbapenem resistant isolate genomes (n = 11) were assembled using the following pipeline. All tools were used with default settings unless stated otherwise. Firstly, TrimGalore v0.6.10 (Krueger *et al.*, 2023) was used to trim and remove any adaptors from our sequences (with Cutadapt v0), they were then quality checked with

FastQC v0.12.1(Krueger *et al.*, 2023). Hostile v.0.4.0 (Constantinides, Hunt and Crook, 2023) was run to remove any human contaminants. The BSI isolates were then assembled using Unicycler v0.4.8 (Wick *et al.*, 2017) with default paired-end settings. The genomes were then sequence-typed using MLST v2.23.0 (https://github.com/tseemann/mlst) and run against the PubMLST database (https://pubmlst.org/) to evaluate sequence clonal groups. Platon v1.7 (Schwengers *et al.*, 2020) was used to separate chromosomal and putative plasmid DNA. The genomes were then annotated using Prokka v1.11 (Seemann, 2014) and Bakta v1.91 (Schwengers *et al.*, 2021). Genomes were quality checked with CheckM v1.2.2 (Parks *et al.*, 2015) against the relevant species database. Completeness scores of 90-95% were accepted for further analysis.

Evaluating Genome Characteristics and Resistance Mechanisms

ABRicate v1.0.1(https://github.com/tseemann/abricate) was used to compare the BSI genome contigs against the following databases: The Comprehensive Antibiotic Resistance Database (CARD) (Jia *et al.*, 2017), Virulence Factor Database (VFDB) (Chen *et al.*, 2016), PlasmidFinder Database (PFDB) (Carattoli *et al.*, 2014), and the Antibacterial Biocide and Metal Resistance Genes Database (BacMet) (Pal *et al.*, 2014) with modified settings as described previously (Leigh *et al.*, 2022). ABRicate was run with an identity of 50-100%, allowing for full homolog detection and reduction of false negatives. R-studio (https://posit.co/download/rstudio-desktop/) was used to create a heatmap that visualized any antimicrobial resistant genes present within the isolates. The heatmap was created from running a script available at github (https://github.com/BioRRW/AMR-and-Virulence-factor-visualization-in-R/blob/main/Heatmap-Clustering.R#L99). The script was modified to fit our data. The heatmap was hierarchically clustered using the Ward D method (Nielsen, 2016), isolates (columns) were clustered using the Euclidean distance method, while genes (rows) were clustered using the binary Jaccard distance method.

Comparing replicon types and investigating genome similarity between sanitary ware isolates.

Sankey Diagrams were created with Flourish (https://flourish.studio/) to compare replicon types between ten *C. freundii* and one *E. coli*. The sanitary ware isolates were collected from Hospital 2 in 2021 and stored in a -80 cyrofreezer for further analysis. Comparisons between isolates were performed in 2024. MASH V2.3 (Ondov *et al.*, 2016) was used to cluster and evaluate the similarity between chromosomal and putative plasmid genomes. MASH correlates with the average nucleotide identity (ANI). To measure the distance between genomes the k-mer size of 21 with a 10,000 MinHash sketch was used, in line with previous work (Abram *et al.*, 2021). Significant data with distances ≤0.05 with a p-value ≤0.05 were reported in a Network analysis created with flourish (https://flourish.studio/).

Results

Swabs Collected from Sanitary Ware

For this study we collaborated with an Irish hospital (Hospital 2) to investigate antimicrobial resistant (AMR) pathogens within hospital sanitary ware (showers, sinks and toilets). Swabs were collected from the showers, sinks, and toilets within rooms A, B, and C once a week for three weeks from March 30th, 2021, to April 13th, 2021. Over two hundred isolates were cultured from swabs collected across the three rooms. Room A contained the most isolates (n = 91) followed by room C (n = 65), and room B (n =59).

Isolate Identification from 16s rRNA Sequencing

Imipenem (8mg/ml) and cefotaxime (4mg/ml) were used to select for isolates resistant to carbapenem and cephalosporin antimicrobials. Isolates were resistant to imipenem (n= 147), and over 60 isolates were resistant to cefotaxime (n = 69), and 30 isolates were

resistant to both imipenem and cefotaxime. Most imipenem resistant isolates (n =131) were identified as *S. maltophilia* (Figure 1), while the most abundant cefotaxime resistant isolates were identified as *C. freundii* (n = 64) (Figure 1). *E. coli* and *Diaphorobacter nitroreducens* were identified the least. *E. coli* (n = 1), Enterobacter spp. (n = 4), and *Citrobacter* spp. (n = 25) isolates were resistant to both imipenem and cefotaxime.

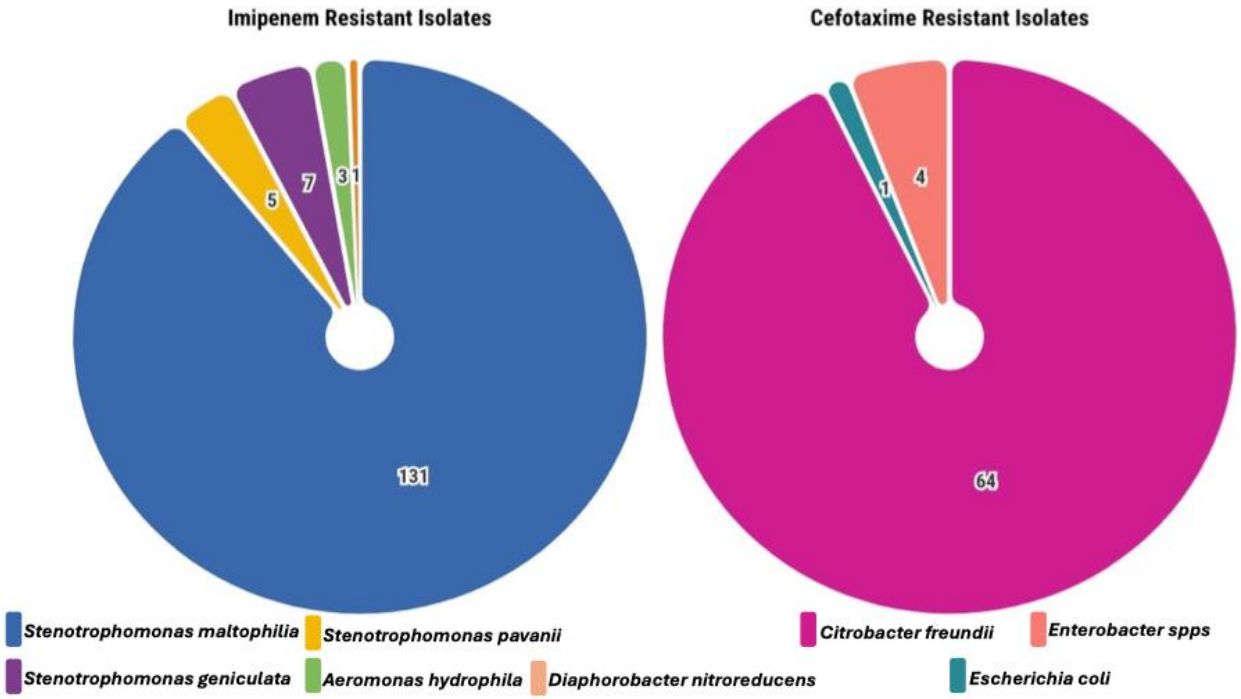


Figure 2.1: Sanitary Ware isolate identification via 16S rRNA sequencing. Isolates that were selected for imipenem resistance are featured in the left pie chart, while isolates that were selected for cefotaxime resistance are featured in the right pie chart. The number of isolates assigned to each species is written in each color segment.

Isolates present within Hospital Sanitary Ware

Imipenem resistant *S. maltophilia* and all cefotaxime Enterobacteriales isolates (n = 69) were chosen for further analysis. Most isolates were selected in room A and were a mix of *S. maltophilia* and *C. freundii* (Figure 2). *Stenotrophomonas maltophilia* were the most

abundant species across the three rooms, while *Enterobacter* species were present in room C only. *Escherichia coli* was present in room A only. Of the three rooms, room B contained the highest number of *S. maltophilia* isolates (n = 52). Room A contained the highest number of *C. freundii* (n = 36).

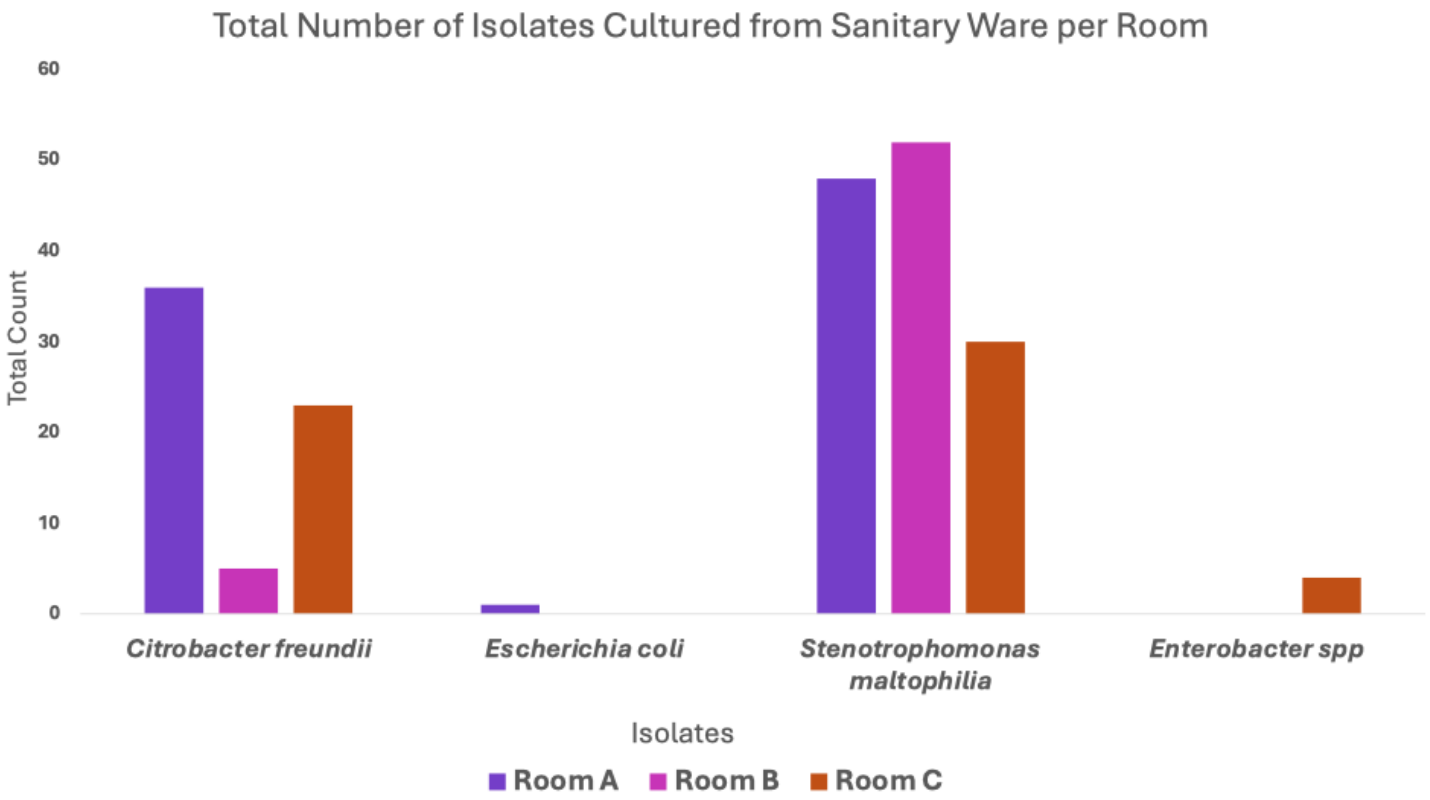


Figure 2.2: The total number of isolates cultured from sanitary ware per room. The isolates were cultured from swabs collected from rooms A, B, and C. The colored bars represent the different rooms.

Isolates were present in all sanitary ware across the three rooms (Figure 3). The isolates were most abundant within the showers (Figure 3) (n = 138), followed by the sinks (n = 45) and toilets (n = 15). *Stenotrophomonas maltophilia* were the most dominant species

present across the showers and sinks. *Citrobacter freundii* were frequently identified from the shower swabs of rooms A and C.

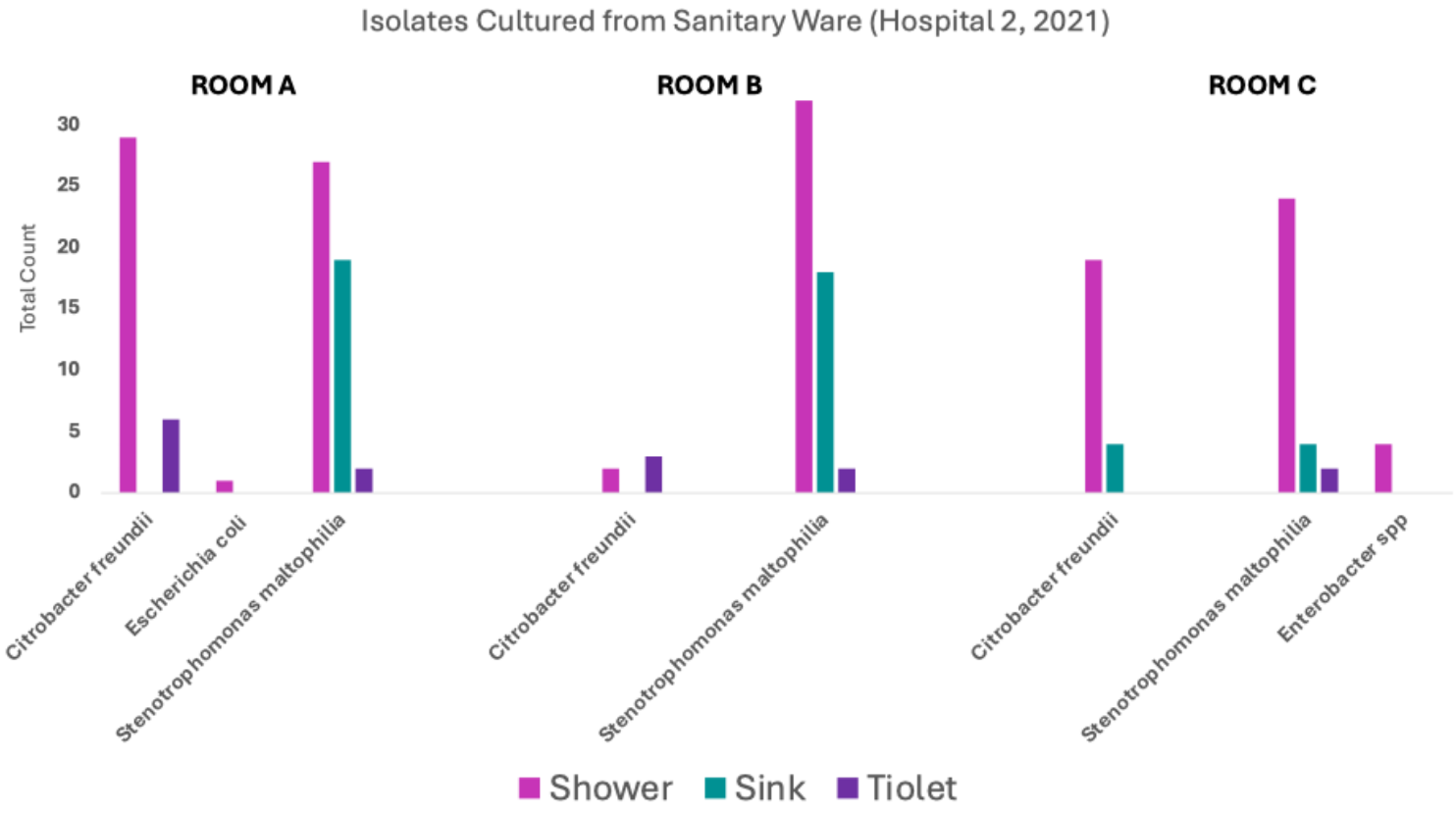
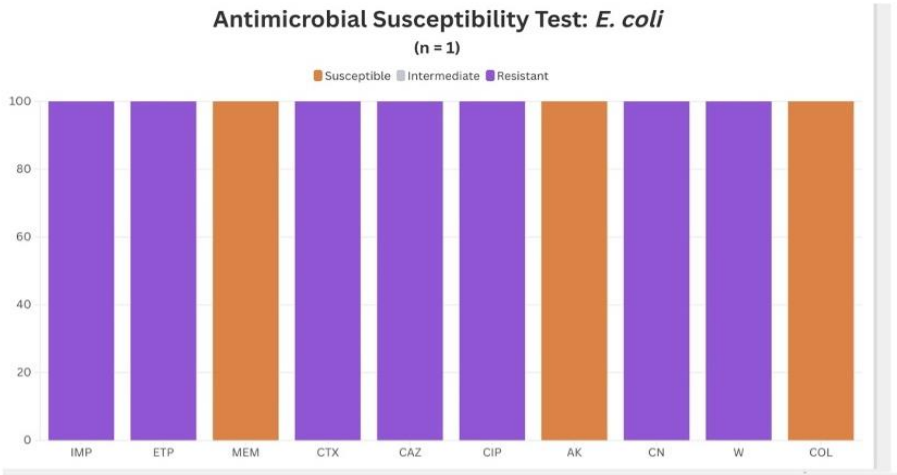


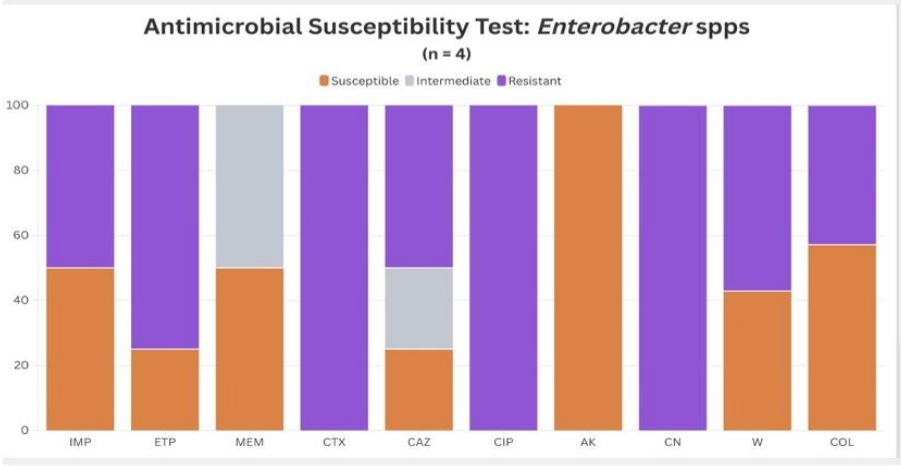
Figure 2.3: The total number of isolates present within the sanitary ware of rooms A, B, and C. The colored bars represent the showers, sinks, and toilets across the three rooms.

Antimicrobial Susceptibility Testing

The table with all AST results can be found in Appendix 1. All isolates selected on cefotaxime were resistant to cefotaxime (Figure 4). The cefotaxime resistant *C. freundii* isolates (n = 64) reported high levels of resistance to gentamicin (98%), trimethoprim (95%), ciprofloxacin (90%), ertapenem (89%), and ceftazidime (85%). Imipenem resistance was reported in 45% and colistin and amikacin resistance was reported in 33% of these *C. freundii* isolates. Meropenem was the most effective antimicrobial tested against these isolates as 83% of isolates were susceptible to the antimicrobial. There were no isolates reporting resistance against meropenem but 17% of isolates were intermediate (Figure 4).

Of the four Enterobacter isolates identified there were high levels of resistance to ceftazidime (100%) and gentamicin (100%). Ertapenem and colistin resistance was detected in 75% of isolates (Figure 4), while imipenem resistance was identified in 50% of isolates. All isolates were susceptible to amikacin and trimethoprim. One *E. coli* isolate was identified, and the antimicrobial susceptibility profile is described in Figure 4 for this isolate.





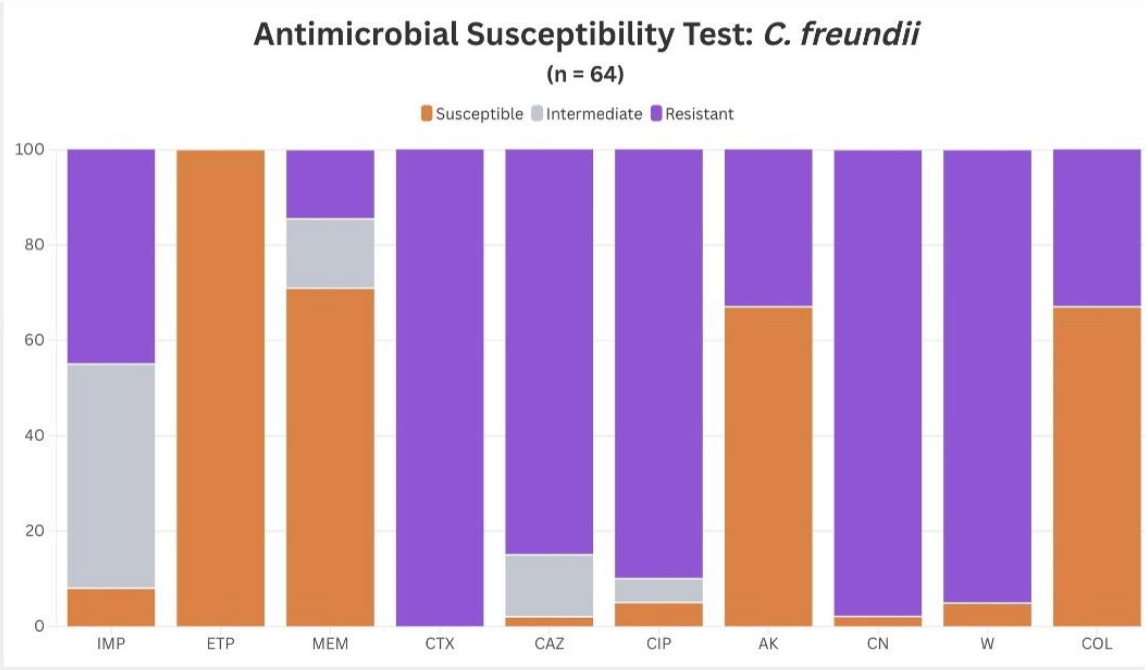


Figure 2.4: Antimicrobial susceptibility tests for *C. freundii* (n = 64)*, Enterobacter* spps (n = 4), and *E. coli* (n = 1) sanitary ware isolates. Each bar represents the overall percentage of susceptible (orange), intermediate (gray), and resistant (purple) rates for each set of isolates. (IMP = Imipenem, ETP = Ertapenem, MEM = Meropenem, CTX = Cefotaxime, CAZ = Ceftazidime, CIP = Ciprofloxacin, AK = Amikacin, CN = Gentamicin, W = Trimethoprim, COL = Colistin).

As *S. maltophilia* are intrinsically resistant to carbapenem antimicrobials both imipenem and meropenem were not effective against these isolates (n =131). All *S. maltophilia* were susceptible to minocycline and levofloxacin antimicrobials, while trimethoprim/sulfamethoxazole was effective against 78% of isolates.

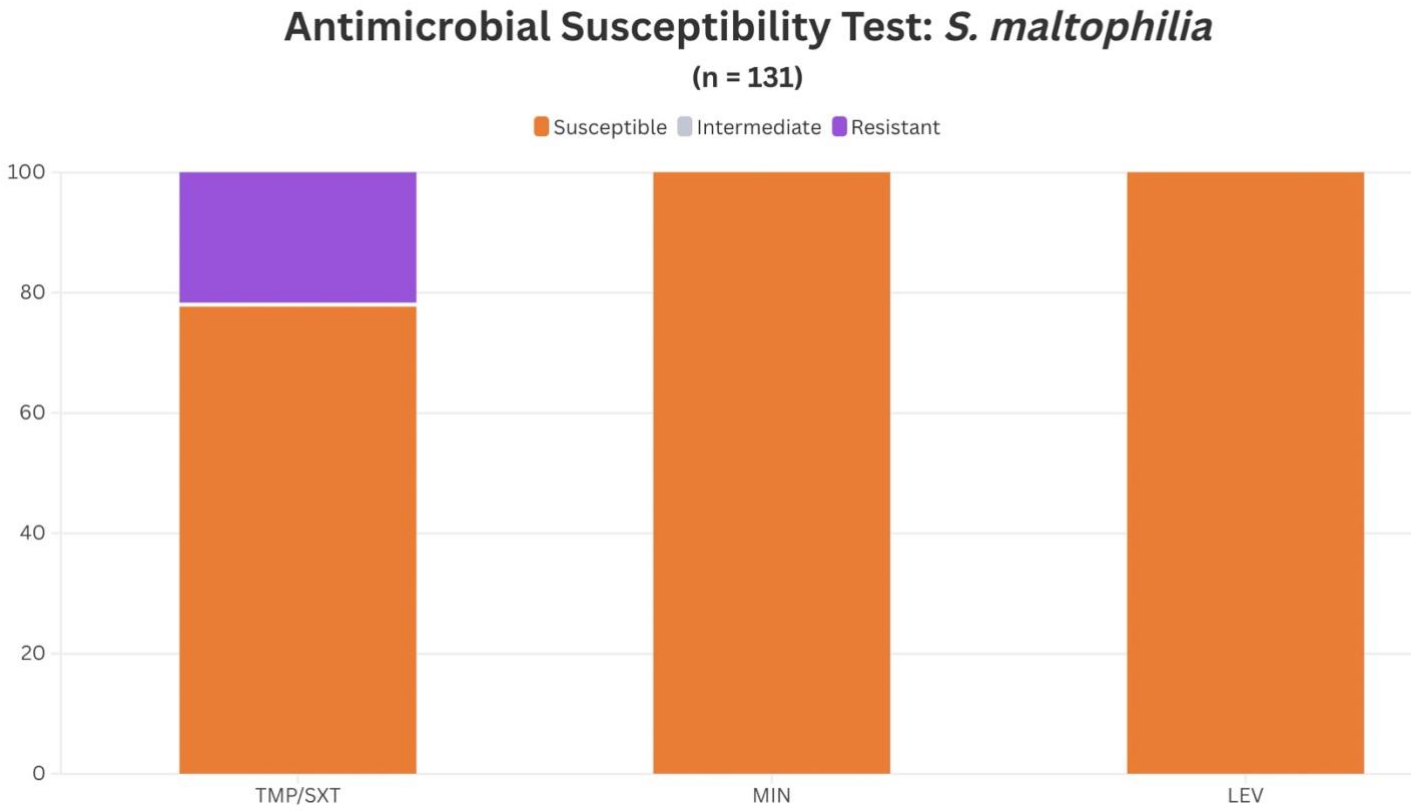


Figure 2.5: Antimicrobial Susceptibility Test: *S. maltophilia* sanitary ware isolates. The bars represent the percentage susceptibility (orange) and resistance (purple) rates for each isolate. (TMP/SXT = Trimethoprim/Sulfamethoxazole, MIN = Minocycline, Lev = Levofloxacin).

Isolate Transmission Within Sanitary Ware at Different Timepoints

In room A, isolates were identified most frequently at the timepoint (TP) 3 (n = 37), while in rooms B and C isolates were present most frequently at TP2 with 24 reported from room B, and 26 reported from room C (Table 2). Across all three timepoints isolates were detected the least from toilets and the most from showers.

In room A, *S. maltophilia* isolates were absent within the toilet for TP1 and present in toilets for TPs 2 and 3 (Table 2). Overall, the numbers of *S. maltophilia* increased slightly from TP1 to TP3. The same pattern of isolation existed for the *C. freundii* isolates, which increased within room A from TP1 to TP3. In the sanitary ware of room B, *S. maltophilia* isolates decreased from TP1 to TP3, and were present in toilets at TP2 only. *S. maltophilia* isolates increased in abundance within the sinks from TP1 to TP3 and decreased within the showers. The *C. freundii* isolates were present in the toilet and shower at TP2 only (Table 2) and absent in all sanitary ware for TP1 and TP3 in rooms B and C. Most isolates were present at TP2 within the shower only (n = 26) in room C. *S. maltophilia* isolates were reported within all sanitary ware locations at TP3, but the numbers of isolates decreased within the shower compared to the those at TP2. *Enterobacter* spps were present within the shower at TP1 and TP2. *C. freundii* isolates were present in the shower at TP 1 and 2, *C. freundii* isolates were present within the sink only at TP3 (Table 2).

Table 2.1: The total number of isolates cultured from sanitary ware at different timepoints across the three rooms. The number represents the total count of isolates cultured from each location; zero indicates that no isolate was cultured.

Room A

TP1 (30/03/2021)	***Stenotrophomonas maltophilia***	***Citrobacter freundii***	***Enterobacter spps***	***Escherichia coli***
Shower	10	8	0	0
Toilet	0	0	0	0
Sink	4	0	0	0
TP2 (07/04/2021)				
Shower	10	9	0	0
Toilet	1	0	0	0
Sink	6	0	0	0
TP3 (13/04/2021)				
Shower	7	12	0	1
Toilet	1	7	0	0
Sink	9	0	0	0

Room B

TP1 (30/03/2021)	***Stenotrophomonas maltophilia***	***Citrobacter freundii***	***Enterobacter spps***	***Escherichia coli***
Shower	14	0	0	0
Toilet	0	0	0	0
Sink	5	0	0	0
TP2 (07/04/2021)				
Shower	12	2	0	0
Toilet	2	3	0	0
Sink	5	0	0	0
TP3 (13/04/2021)				
Shower	5	0	0	0
Toilet	0	0	0	0
Sink	8	0	0	0

Room C

TP1 (30/03/2021)	***Stenotrophomonas maltophilia***	***Citrobacter freundii***	***Enterobacter spps***	***Escherichia coli***
Shower	4	7	3	0
Toilet	0	0	0	0
Sink	1	0	0	0

TP2 (07/04/2021)				
Shower	15	10	1	0
Toilet	0	0	0	0
Sink	0	0	0	0
TP3 (13/04/2021)				
Shower	5	2	0	0
Toilet	2	0	0	0
Sink	3	4	0	0

Genotypical and Phenotypical Characteristics

Eleven AMR pathogens were chosen to undergo whole genome sequencing. These isolates were selected as they displayed high phenotypical resistance levels to cefotaxime, ciprofloxacin, gentamycin, and trimethoprim antimicrobials. Some of these isolates (n = 8) were resistant to ertapenem and (n = 7) imipenem. Of the eleven isolates, ten were sequence typed as *C. freundii* ST116, while one was sequence typed as *E. coli* ST131. Isolates were mainly reported within the shower (n = 9) followed by the toilet (n = 2). Isolates were present within rooms A and B, no isolates were present in room C. Cefotaxime resistance was reported in 100% (n = 11) of isolates with nine of these isolates present in room A (82%) and two present in room B (18%). Ertapenem resistance was reported in 73% (n = 8) of isolates (Table 3), with the two isolates from room B displaying ertapenem resistance and six from room A. There was an average of 54 antimicrobial resistant genes (ARG) reported within each isolate (Table 3). The carbapenemase bla_{OXA-48} gene was present within 64% of isolates (n = 7), this gene was present within the two isolates from room B, and five isolates from room A.

Table 2.2: Phenotypic and Genotypic characteristics of eleven sanitary ware isolates. Date refers to each timepoint. TP1 (30/3/21) TP2 (07/04/21) and TP3 (13/ 04/21).

ID	ISOLATE	HOSPITAL	DATE	ROOM	LOCATION	ST	RES PHENOTYPE	# ARG	CPE GENE(S)
CF9	*Citrobacter freundii*	2	30/03/2021	A	Shower	ST116	ETP, CTX, CIP, CN, W	53	
CF1	*Citrobacter freundii*	2	07/04/2021	A	Shower	ST116	CTX, CAZ, CN, W, CIP	53	
CF2	*Citrobacter freundii*	2	07/04/2021	B	Shower	ST116	IMP, ETP, CTX, CAZ, CIP, AK, CN, W	54	*blaOXA-48*
CF4	*Citrobacter freundii*	2	07/04/2021	A	Shower	ST116	IMP, ETP, CTX, CAZ, CIP, AK, CN, W	54	*blaOXA-48*
CF5	*Citrobacter freundii*	2	07/04/2021	B	Shower	ST116	IMP, ETP, CTX, CAZ, CIP, AK, CN, W	54	*blaOXA-48*
CF10	*Citrobacter freundii*	2	07/04/2021	A	Shower	ST116	IMP, ETP, CTX, CAZ, CIP, CN, W	54	*blaOXA-48*

ID	ISOLATE	HOSPITAL	DATE	ROOM	LOCATION	ST	RES PHENOTYPE	# ARG	CPE GENE(S)
CF11	*Citrobacter freundii*	2	07/04/2021	A	Shower	ST116	IMP, ETP, CTX, CAZ, CIP, AK, CN, W	54	*blaOXA-48*
CF6	*Citrobacter freundii*	2	13/04/2021	A	Shower	ST116	CTX, CAZ, CIP, AK, CN, W	53	
CF7	*Citrobacter freundii*	2	13/04/2021	A	Toilet	ST116	IMP, ETP, CTX, CAZ, CIP, CN, W	54	*blaOXA-48*
CF8	*Citrobacter freundii*	2	13/04/2021	A	Toilet	ST116	CTX, CAZ, CIP, AK, CN, W	53	
EC3	*Escherichia coli*	2	13/04/2021	A	Shower	ST131	IMP, ETP, CTX, CAZ, CIP, AK, CN, W	54	*blaOXA-48*

ST = sequence type, RES PHENOTYPYE = antimicrobial resistance phenotype, # ARG = total number of antimicrobial resistant genes found within the isolate, CPE GENES = carbapenemase producing genes present.

Antimicrobial, Metal and Biocide Resistance Genes and Virulence Factors

On average there were 5 antimicrobial resistant genes present on plasmid contigs within each isolate. Of these genes, *bla*$_{OXA-48}$ (Figure 6) was reported on plasmid contigs of 64% of isolates (n = 7), followed by *bla*$_{TEM-33}$ on plasmid contigs of 91% of isolates (n = 10), and *bla*$_{OXA-10}$, *cmlA1*, *sul1*, *ANT (2")-Ia* on the plasmid contigs of all isolates (n = 11). All other antimicrobial resistant genes reported were present on chromosome contigs, with *arnA*, *bla*$_{TEM-1}$, and *CMY-84* reported within *Escherichia coli* ST131 only. The genes, *bla*$_{TEM-33}$ and *CMY-135* were present in the *Citrobacter freundii* ST116 isolates. The fluroquinolone resistant *QnrA3* gene was present in all isolates in addition to the *dfrA1*- which results in trimethoprim resistance.

Antimicrobial Resistant Genes (ARGs) Presence/ Absence within Sanitary Ware Isolates

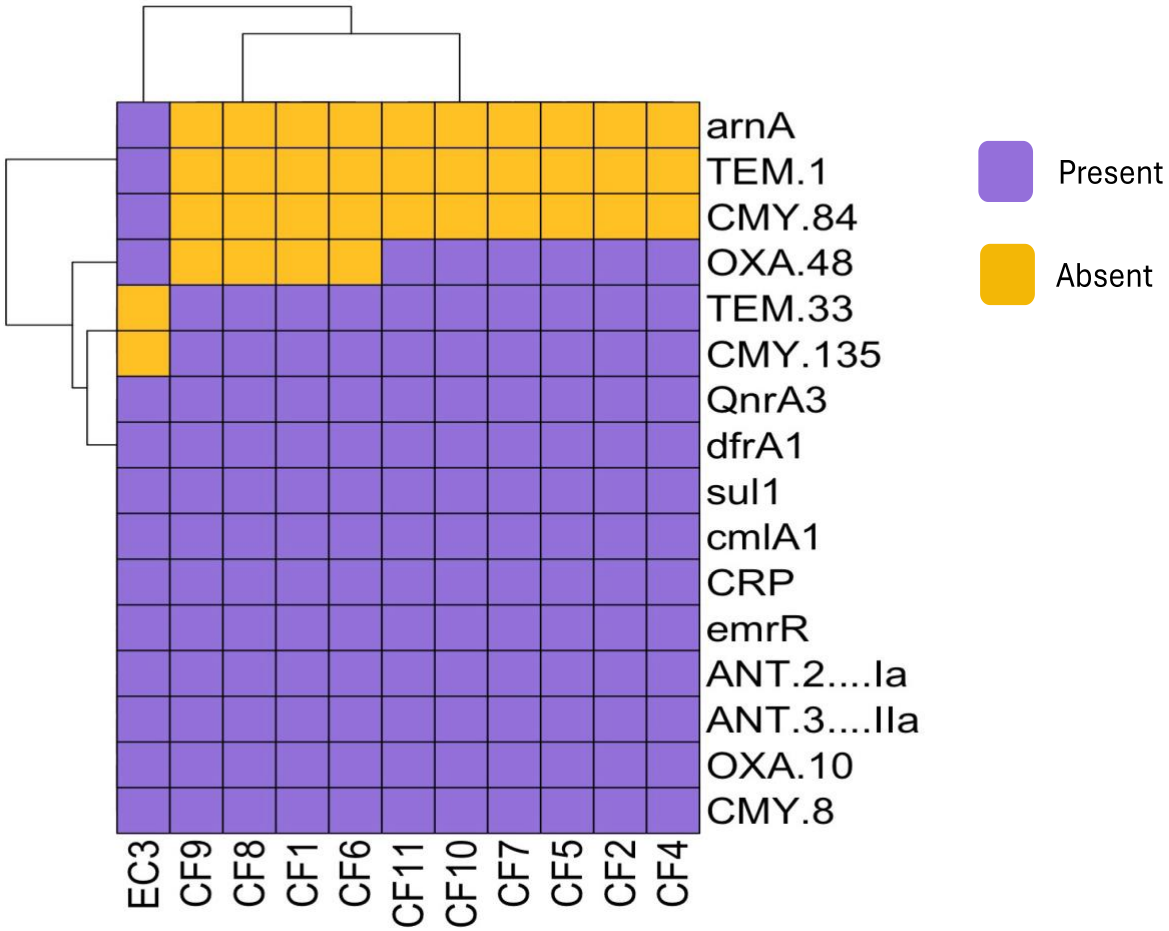


Figure 2.6: Heatmap displaying antimicrobial resistant genes (ARGs) present/ absent within eleven sanitary ware isolates. Columns (isolates) were clustered using the Euclidean distance and are displayed horizontally on the heatmap. Rows (ARGs) were clustered using Euclidean distance and are displayed vertically on the heatmap. Ward's clustering method was used to hierarchically cluster the heatmap. Purple boxes represent the gene reported present within the isolate and golden boxes report the gene being absent.

On average there were 139 MRGs reported on chromosomal contigs and 18 MRGs reported on plasmid contigs within the genome of each isolate. The mercury resistant operon (*merA/B/C/D/E/P/R/R2/T*) was reported on plasmid contigs of all isolates, in addition to *qacEdelta1,* and the tellurium resistant operon (*terA/B/C/D/E/W/Z)*.

Present on chromosomal contigs within the bacterial isolates were MRGs such as *acrF-envD* and *acrE-envC* which encode efflux pumps, which may cause resistance to multiple metal and biocide compounds like sodium dodecyl sulfate (SDS), acriflavine, sodium deoxycholate, sodium cholate and sodium taurodeoxycholate. *Citrobacter freundii* isolates all contained arsenic resistant genes (*arsB/C/H*), which were absent in *E. coli* ST131 (Figure 7).

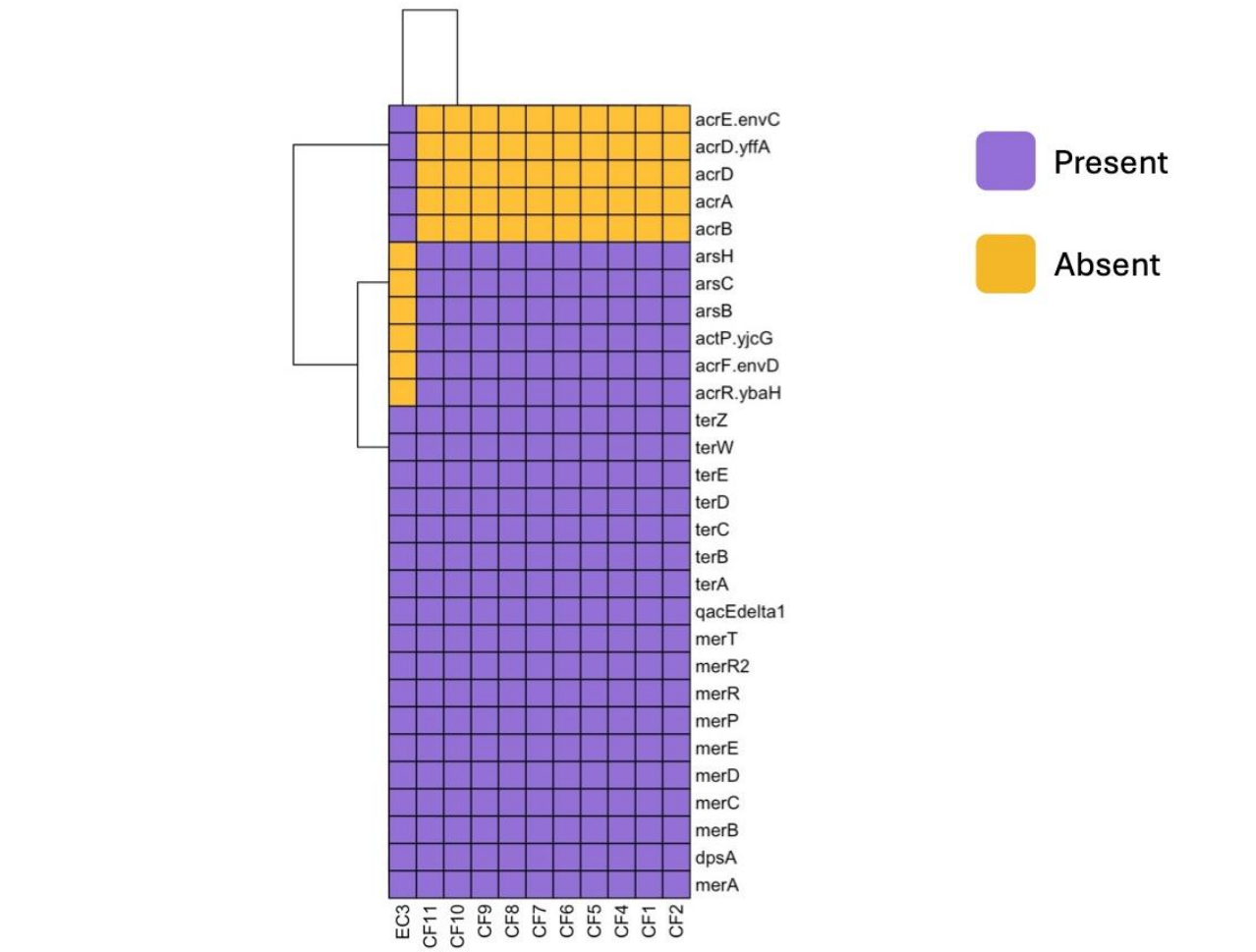


Figure 2.7: Heatmap displaying metal resistant genes (MRGs) present/ absent within eleven sanitary ware isolates. Columns (isolates) were clustered using the Euclidean distance and are displayed horizontally on the heatmap. Rows (MRGs) were clustered using Euclidean distance and are displayed vertically on the heatmap. Ward's clustering method was used to hierarchically cluster the heatmap. Purple boxes represent the gene reported present within the isolate and golden boxes report the gene being absent.

There was an average of 68 virulence factors present on the chromosomes of each isolate (Figure 8). No virulence factors were detected on the plasmid contigs. Of the virulence factors, adhesins such *as fimH, fimD, fimF, fimC,* and *fimL* were present in all isolates.

Proteins involved in chemotaxis*, cheW, cheZ*, and *cheY* were present in all isolates, while *cheD* was present in all *Citrobacter freundii* isolates. Protectins like *ompA*, *entE*, and *entF* were reported in all isolates. *Escherichia coli ST131* contained virulence factors *rfaE, luxS, fihB, figD, chuS*, and *chuY* which were not present in any *C. freundii* ST116 isolate. Similarly, *rffG, cheD, shuY*, and *shuS* were reported within all *C. fruendii* ST116 isolates but absent within *E. coli* ST131.

Virulence Factors Presence/ Absence within Sanitary Ware Isolates

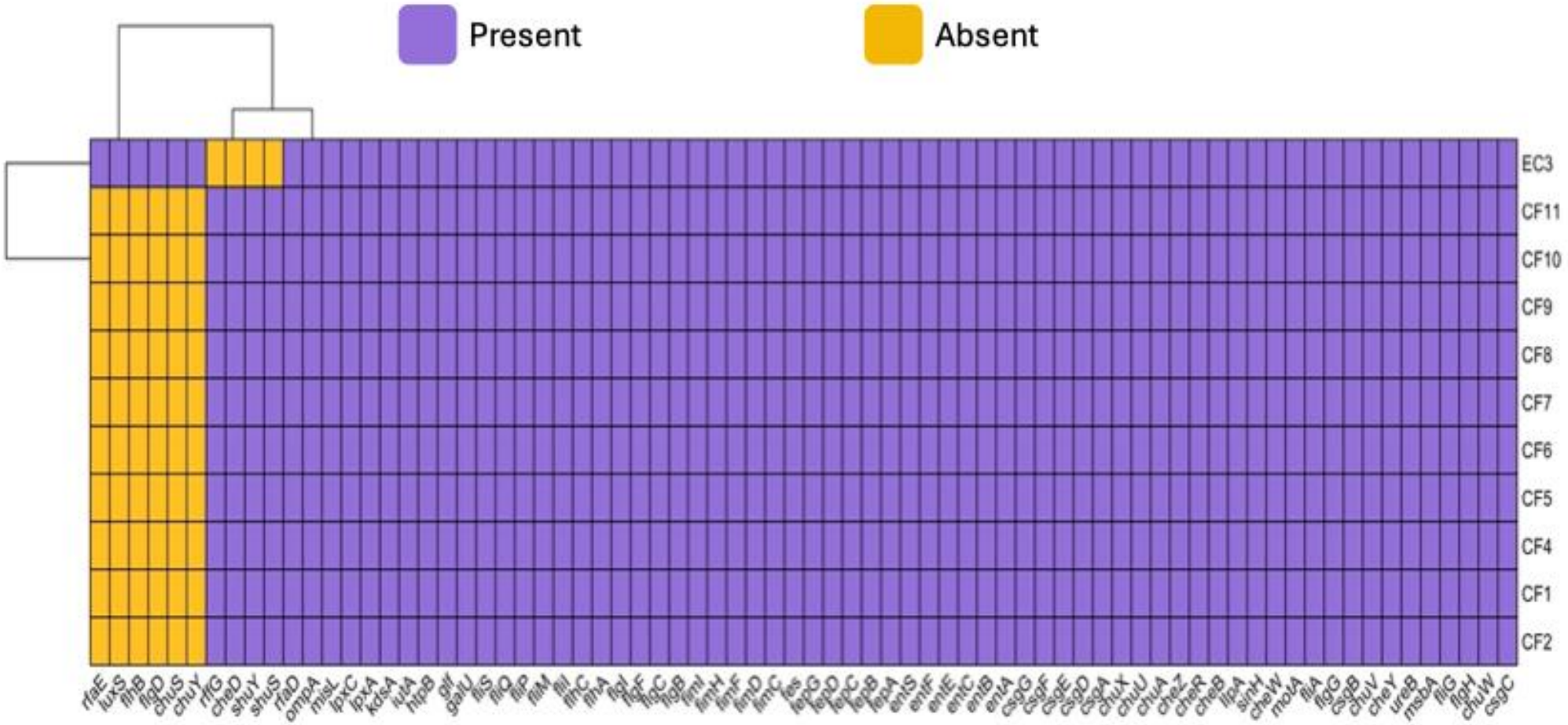


Figure 2.8: Heatmap displaying virulence factors present/ absent within eleven sanitary ware isolates. Columns (virulence genes) were clustered using the Euclidean distance and are displayed horizontally on the heatmap. Rows (samples) were clustered using Euclidean distance and are displayed vertically on the heatmap. Ward's clustering method was used to hierarchically cluster the heatmap. Purple boxes represent the gene reported present within the isolate and golden boxes report the gene being absent.

Plasmid Replicon Types

On average there were four plasmid replicon types reported within each isolate. Of these replicon types, IncA/C2_1, IncH1A (CIT_1_pNDM-CIT, and IncHIB (CIT)_1_pNDM-CIT co-occurred across all eleven isolates. IncL/M(pOXA-48) _1_pOXA-48 occurred within seven isolates (six *C. freundii* and one *E. coli*), the same seven that carried the bla_{OXA-48} gene. This replicon type co-occurred with IncA/C2_1, IncH1A (CIT_1_pNDM-CIT, and IncHIB (CIT)_1_pNDM-CIT within seven isolates. One isolate contained all five replicon types highlighted (Figure 9). Over 70 % of isolates carrying the IncL/M(pOXA-48) _1_pOXA-48 replicon type was collected from room A (n =5) while the remaining 18% were collected from room B (n =2).

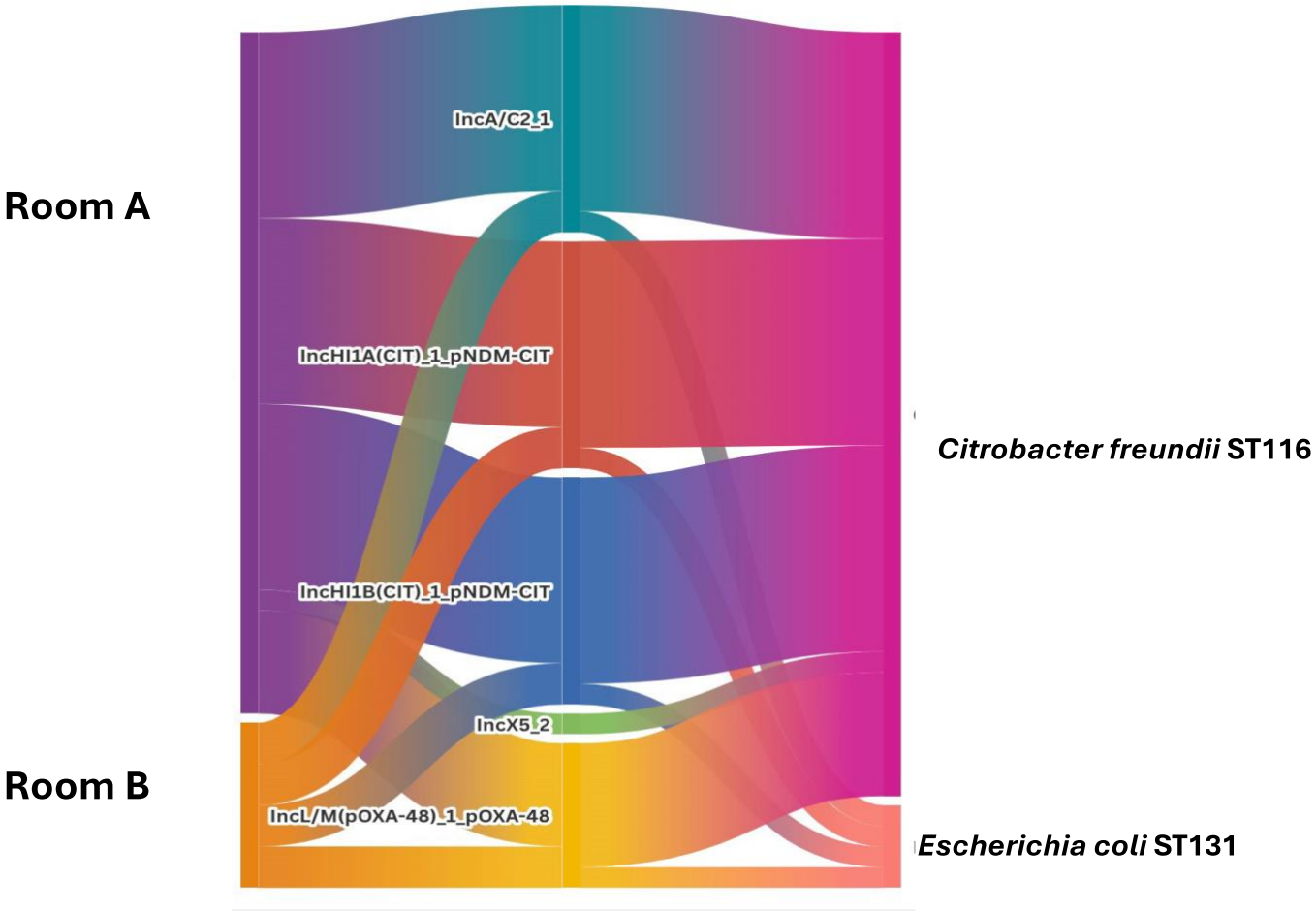


Figure 2.9: Sankey diagram displaying plasmid replicon types occurring within the hospital sanitary ware isolates per rooms A, and B. There were no isolates present from room C. The bars on the left represent the two rooms, the bars in the middle display the different replicon types, and the bars on the right are the sanitary ware isolates grouped by their sequence type.

Comparative genome analysis via MASH distancing.

Isolates collected from room A contain a purple point, while isolates collected from room B are orange. Analysis of the chromosomal genomes indicates that the *C. freundii* ST116 isolates group together, while the *E. coli* isolate is an outlier (Figure 10). *Citrobacter freundii* ST116 isolates shared low distance values and similarity rates ranging from 99.3 to 100% depending on which of the isolates were compared against each other. *Escherichia coli* and the *C. freundii* isolates shared higher distance values which was expected as they're different species and their chromosomes differ more than their plasmid genotypes.

When we looked at how related to each other the plasmids where, we seen that all isolates share a similar plasmid genotype regardless of species, room, or location. There were some replicon types - IncA/C2_1, IncH1A (CIT_1_pNDM-CIT, and IncHIB (CIT)_1_pNDM-CIT – co-occurring across all the isolates (Figure 10). Isolates carrying the IncL/M_(pOXA-48) _1_pOXA-48 or IncL plasmid replicon type were of particular focus as this replicon type has been previously associated with bla_{OXA-48}, which was present in the same isolates carrying this plasmid replicon type. The distances between the isolates carrying this IncL plasmid were low, and their similarity rates ranged from 95 to 100%.

The IncL plasmid was present within both *C. freundii* and *E. coli* isolates and these isolates were collected from separate rooms (A and B), and locations (shower, toilets). Some of the isolates carrying this plasmid were collected at three different timepoints – with one isolate reported at timepoint 1, six reported at timepoint 2, and four of the isolates reported at timepoint 3. From the current isolates, the IncL plasmid replicon type appears within *E. coli* and *C. freundii* isolates at timepoint 2 (7/4/21) and timepoint 3 (13/4/21) this replicon type is not present at timepoint 1 (30/3/21).

Interestingly, there were two *C. freundii* (CF2, CF10) isolates that have a high probability of being identical as they shared 100% similarity between their chromosomal and plasmid genotypes, which is surprising as these isolates were collected from different

rooms, but the same location, the shower. Long read sequencing and more analysis is needed to confirm the isolates are 100% identical.

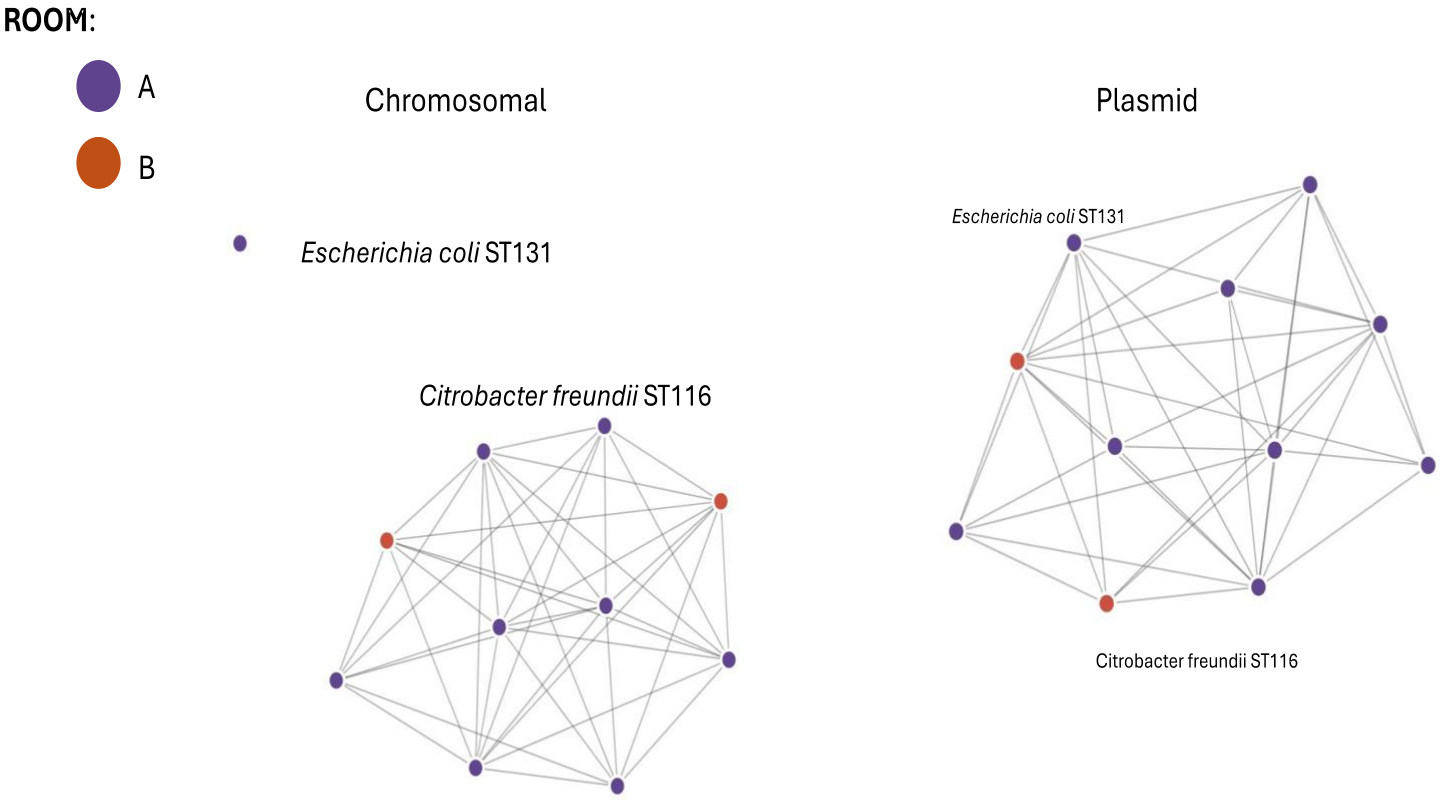


Figure 2.10: Chromosome and plasmid genome relatedness via MASH distancing network graphs. The lower the distance the more related the genomes. The colored dots represent the rooms, with purple for any isolate collected from room A, and orange for any isolate collected from room B. The network graph on the left refers to the chromosome genomes, while the network graph on the right contains the putative plasmid genomes.

Discussion

This study highlights that Irish hospital sanitary ware act as reservoirs for AMR pathogens, particularly ESBL and CP producing bacteria, reveals the genotypic profile of some ESBL and CP producing *Citrobacter* isolates, and found a large presence of *S. maltophilia* populations within Irish hospital sanitary ware. The findings of this study provide significant information on the microbial environment within sanitary ware and may assist in the management of these microbes. Global studies have reported high CP and ESBL producing bacterial populations present within toilets (Heireman *et al.*, 2020; Kim *et al.*, 2022; Valzano *et al.*, 2024; Larsen *et al.*, 2025), while our study found that the toilets harboured the least number of isolates. In line with the Health Protection Surveillance Centre CPE reports, 2021 (Enhanced Surveillance of Carbapenemase-Producing Enterobacterales (CPE), 2021) and 2023 (Enhanced Surveillance of Carbapenemase-Producing Enterobacterales (CPE), 2023), we found the showers held the highest number of CP and ESBL producing isolates.

Large levels of the CP producing bacteria were identified as *S. maltophilia*. *Stenotrophomonas maltophilia* is commonly isolated from water-based environments (Brooke, 2012) and intrinsically express the carbapenemase enzymes, L1 and L2 (Hu *et al.*, 2008; Mojica *et al.*, 2022; Bhaumik, Aungkur and Anderson, 2024), which provide them with protection against almost all beta-lactam antimicrobials. One study reported this bacterium within Irish drinking water (Alawi *et al.*, 2024) and like our study another reported *S. maltophilia* present in Irish hospital showers and sinks (Wu *et al.*, 2025). Reports of *S. maltophilia* remain low and this could be reasoned that even though *S. maltophilia* was the most dominant bacterium isolated throughout this study, the rate of *S. maltophilia* infections across European acute hospitals, including Ireland, are less than 1% (0.6 % for surgical site infections, 0.1 % for UTIs, and 0.7 % for Bloodstream infections) (European Centre for Diseases Prevention and Control, point prevalence survey of healthcare associated infections and antimicrobial usage in European acute hospitals, 2024). In comparison, it is noted that a meta-analysis reported by Banar *et al.*,

2023, which analyzed the prevalence of *S. maltophilia* infections throughout the Western-Pacific and Americas regions and compared the trends of antibiotic resistance of *S. maltophilia* before and after 2010 concluded there is a slow increase of *S. maltophilia* infections occurring across these regions. In terms of AMR treatment, trimethoprim-sulfamethoxazole remained the most effective against *S. maltophilia*.

As previous studies have highlighted *S. maltophilia* rarely infect immunocompetent patients, in contrast, these bacteria are problematic in immunocompromised patients, especially those with cystic fibrosis (Waters *et al.*, 2011; Stanojevic *et al.*, 2013; Terlizzi *et al.*, 2023). Ireland has the highest rates of cystic fibrosis across Europe (Gabbi, Renieri and Strandvik, 2022) and though reports from the Irish annual cystic fibrosis registry did not find any *S. maltophilia* present in patients from 2021 (Cystic fibrosis registry of Ireland, 2021) they appear at low rates (<3%) in 2022 (Cystic fibrosis registry of Ireland, 2022) and 2023 (Cystic fibrosis registry of Ireland, 2023). These rates are like those in other European countries (ECFSPR Annual Report, 2022). *Stenotrophomonas maltophilia* infections are difficult to treat due to the lack of standardized breakpoints limiting antimicrobial options, and their intrinsic resistance to broad-spectrum antimicrobials (Denton and Kerr, 1998; Looney, Narita and Mühlemann, 2009; Aysert-Yıldız *et al.*, 2022). Trimethoprim/Sulfamethoxazole (TMP/SMX) is the drug of choice against these bacteria (Falagas *et al.*, 2008; Sarzynski *et al.*, 2022). Studies mapping antimicrobial resistance rates within *S. maltophilia*, reported low levels of TMP/SMX resistance across Europe (<15%) (Banar *et al.*, 2023; Dadashi *et al.*, 2023; Bostanghadiri *et al.*, 2024). Concerningly, some isolates (22%/29) in our study reported TMP/SMX resistance, though it is a small increase in comparison to a previous study by Livermore *et al.*, 2008, in which all their *S. maltophilia* isolates were universally susceptible to TMP/SMX.

The prevalence of our ESBL producing bacteria isolated from hospital sanitary ware was higher (n = 69) in comparison to a previous study (Morris *et al.*, 2003) Like the CP producing isolates, the showers contained the highest number of ESBL producing isolates, though in comparison, we found more CP producing bacteria than ESBL producing bacteria. ESBL enzymes are frequently produced within the Enterobacterales

species (Paterson, 2006; Coque, Baquero and Cantón, 2008; Dirar *et al.*, 2020). Previous reports have highlighted large collections of ESBL- producing *E. coli* and/ or *K.* pnemouniae within European hospital environments (Pilmis *et al.*, 2018; Van Den Bijllaardt *et al.*, 2018; Vink, Edgeworth and Bailey, 2020), and similar results reported within Irish hospital environments (Skally *et al.*, 2014; O'Connell *et al.*, 2015). In contrast, the largest population of ESBL- producing isolates in our study were identified as *C. freundii*. In comparison to *E. coli* and *Klebsiella*, *Citrobacter* are a lesser studied species within the Enterobacterales although global studies have reported this bacterium residing within hospital sanitary ware (Jolivet *et al.*, 2021; Hamerlinck *et al.*, 2023), and it is linked to the transmission of both ESBL and carbapenemase genes, notably the Class D carbapenemase, bla_{OXA-48} (Kanamori *et al.*, 2011; De Geyter *et al.*, 2017; Tariq *et al.*, 2023; Sharew *et al.*, 2025). Infection rates of ESBL-producing *Citrobacter* across European acute hospitals were higher in comparison to CP producing *Citrobacter* rates (European Centre for Diseases Prevention and Control, point prevalence survey of healthcare associated infections and antimicrobial usage in European acute hospitals, 2024).

Citrobacter freundii was one of the sub-species of *Citrobacter* resulting in these infections for Irish hospitals, the percentage of these isolates in comparison to the overall number of bacterial isolates reported, remain less than 1%. Few Irish studies have reported on ESBL production within *Citrobacter*. One study reported ESBL-producing *C. freundii* isolates collected from patients within an Irish university hospital, although the ESBL type was not disclosed (Fennell *et al.*, 2012). While another study, isolated one ESBL positive *Citrobacter youngae* from high risk patients within an Irish tertiary care hospital (O'Connell *et al.*, 2015), this isolate did not produce either CTX, TEM or SHV type ESBLs but was an AmpC producer. Another recent study by (Hooban *et al.*, 2022) reported on various ESBL producing Enterobacterales within the Irish environment, with the CTX-type ESBLs dominating across all their samples. We found the TEM- type ESBL, $bla_{TEM-33,}$ was carried by some of our *C. freundii* isolates. Although more research is needed to evaluate which ESBL-type was most prevalent across our isolates as not all our isolates underwent whole genome sequencing.

Citrobacter freundii isolates have been previously reported to harbor bla_{OXA-48} (Tafoukt *et al.*, 2017; Lalaoui *et al.*, 2019; Brehony *et al.*, 2021; Biez *et al.*, 2022; Sommer *et al.*, 2024), and studies across the globe have associated this bacterium with the sequence type, ST22 (Jolivet *et al.*, 2021; Biez *et al.*, 2023; Jabeen *et al.*, 2023). In comparison, some of our *C. freundii* that carried bla_{OXA-48} were sequence typed ST116. Other CP producing *C. freundii* ST116 isolates were reported within clinical studies from Finland (Räisänen *et al.*, 2021), China (Guo *et al.*, 2024), and Italy (Mattioni Marchetti *et al.*, 2024). There are few reports of ST116 *C. freundii* within Ireland, although one study by (Maguire *et al.*, 2025) isolated this bacterium carrying bla_{OXA-48} from human clinical samples. Interestingly, the same study by Maguire *et al*, found the most dominant plasmid replicon type associated with bla_{OXA-48} isolates to be IncL which was similarly the case for our study.

In addition to our isolates reporting intrinsic and adaptive ARGs, we found that the significant MRG, *qacEdelta1*, was harboured by all our sequenced isolates. Previous reports have highlighted the co-occurrence of this MRG and the ARG, *sul1*, in *Citrobacter* isolates (Mori *et al.*, 2021; Kelly *et al.*, 2023). The same co-occurrence can be seen in our study with both genes reported on all plasmid replicon types (n=5) within our isolates (n=11). Concerningly this shows that these genes co-occur with bla_{OXA-48}. This provides the isolates protection against the strongest antimicrobials and with *qacEdelta1*, the bacteria are resistant to disinfectants/antiseptic products. Due to their presence on plasmids these genes have a high chance of being mobile throughout the hospital, increasing the presence of AMR pathogen reservoirs. What's more all plasmids harboured the mercury (*merA/B/C/D/E/P/R/R2/T*) and tellurium (*terA/B/C/D/E/W/Z*) operons, allowing the bacteria to reside in heavy metal areas, our results are similar to a previous Irish rural study (Andrade *et al.*, 2023). Though our isolates are clinical rather than rural. Interestingly, there were no VFs reported on any of the plasmids, meaning that these isolates, while spreading pathogenic genes across the hospital, they don't spread genes that assist in the colonization of the host. There is a chance these isolates can still colonise and infect patients with the report of some adhesin and protectin genes on the chromosomes. Although these genes appear on all isolates (minus the slight AST profile differences between *E. coli* and *Citrobacter*) indicating they are possibly conserved

genes. They are significant for bacterial survival and function, but not as relevant as acquired VFs for the conization and infection of a host.

Conclusion

This study provides evidence of Irish hospital sanitary ware acting as reservoirs for ESBL and CP producing bacteria. Large populations of *S. maltophilia* and *C. freundii* were among the bacteria isolated from sanitary ware although their infection rates remain low across Irish and European hospitals. Evidently for *C. freundii*, this could be due to the absence of acquired VFs among these isolates. While some *Citrobacter* isolates produced bla_{TEM-33} and bla_{OXA-48}, these genes were reported on plasmids which could be spread to pathogens with high levels of VFs, increasing infection indirectly. Although more research is needed to confirm this theory and understand their epidemiology / transmissions across Irish hospitals.

Chapter 3: Comparative genomics of Extended- spectrum-beta-Lactamase-producing Enterobacterales in addition to methicillin-resistance *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium* bloodstream infection isolates cultured within Irish hospitals.

Introduction

Antimicrobial resistance (AMR) is a major public health challenge (Jindal, Pandya and Khan, 2015; Süle, 2022), with the ability to increase patients' stay in healthcare associated facilities while limiting therapeutic efficiency and increasing treatment costs (Ahmed *et al.*, 2024). Globally, over 8 million deaths were reportedly due to bacterial infections in 2019. Approximately 15% of those deaths were caused by AMR, while 61% were associated with AMR (Naghavi *et al.*, 2024).

Bloodstream infections (BSIs) occur due to the presence of pathogens within the blood (Viscoli, 2016). These infections can range from cryptogenic –the BSI manifests alone and are typically treated effectively – or secondary – manifesting with focal infections such as meningitis or pneumoniae (Banik *et al.*, 2018). Bloodstream infections can be especially dangerous for immunocompromised hosts and remain the most dominate form of invasive infections throughout Ireland.

Bloodstream infections can emerge due to the presence of AMR bacteria such as third-generation cephalosporin resistant Enterobacterales like *E. coli* and *Klebsiella pneumoniae,* along with the Gram-positive methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *E. faecium* (VRE). Third-generation cephalosporin resistant *E. coli* carry enzymes known as extended-spectrum-beta-lactamases (ESBLs) which drive their resistance to these antibiotics. Cefotaximase (CTX-M) type genes are the most abundant ESBL-type worldwide (Jorgensen *et al.*, 2010; Yu *et al.*, 2024).

Escherichia coli is known to have pathogenic and non-pathogenic lineages and one pathogenic lineage – ST131 *E. coli* - has been reported as the leading causative agent within community-associated and hospital-associated infections (Kudinha *et al.*, 2013; Decano and Downing, 2019). This extraintestinal pathogenic *E. coli* (ExPEC) has been documented globally owing its dominance to a wide range of virulence factors, antimicrobial resistant genes (ARGs) and mobile genetic elements (MGEs) (Peirano *et al.*, 2010). ST131 *E. coli* have been serotyped into various groups including the newly emerging ST131-Onovel31:H4 (Shawa *et al.*, 2021). These isolates have been reported to

carry *bla*$_{CTX\text{-}M\text{-}15}$ in addition to shiga-toxins and fim-type adhesins. ST131 isolates typically acquire MGEs such as Inc-type conjugative plasmids which contribute to their dissemination.

One significant tool in the fight against these AMR pathogens is whole genome sequencing (WGS) (Oniciuc *et al.*, 2018). Sequencing technologies have improved microbial surveillance, diagnostics, epidemiology studies, and helped with the development of newer antimicrobial therapies (Köser, Ellington and Peacock, 2014; NIHR Global Health Research Unit on Genomic Surveillance of AMR, 2020; Jauneikaite *et al.*, 2023). Research has shown whole genome sequencing as a valuable tool for pathogenic surveillance from blood cultures; identifying pathogens within 24 hours (Peterson *et al.*, 2023). There are species-specific bioinformatic tools available for pathogenic sequence typing, serotyping, or assigning phylogroups. These have expanded the identification of specific strains to include clonal groups or clonal complexes that differ between geographical location, clinical/clinical or clinical/environmental settings.

The objectives of this study were to characterize and compare eighty bloodstream infection isolates received from three different Irish hospitals. The isolates underwent a specific bioinformatic pipeline allowing for the detection of different resistant genes - ARGS, MRGS, and BRGs - in addition to separation and subsequent investigation into the chromosomal and plasmid genotypes of each isolate. This gave us an insight into the movement of these resistance genes and allowed us to see if they were plasmid mediated or intrinsic. The similarities and relatedness between each BSI isolate will was evaluated using a MASH distancing network analysis. This allowed us to visualize the similar / different relationships between the BSI isolates to each other and then across the different Irish hospitals, which is something that hasn't been done yet in Irish clinical studies.

Materials and Methods

Bloodstream Infection Isolate collection and culture methods:

For this study we collaborated with three Irish hospitals. BSI isolates were collected from patients' within each hospital. We requested ESBL and/or CP *E. coli* and *Klebsiella* species, methicillin-resistant *S. aureus* (MRSA), and vancomycin-resistant *E. faecium* (VRE) BSI isolates to be sent to our laboratory for analysis. We further requested any multi-drug resistant (MDR*) E. coli* and *Klebsiella* BSI isolates. All isolates were sent to us cultured on Nutrient Agar slopes and/or plates. ESBL *E. coli* and *Klebsiella* were cultured in Muller-Hinton Broth (DifcoTM Becton, Dickinson and Company, USA) with cefotaxime (4 mg/ml) and carbapenem resistant isolates were cultured in Muller-Hinton Broth with added imipenem (8 mg/ml). *S. aureus* was cultured in Muller-Hinton Broth with added oxacillin (4 mg/ml), and *E. faecium* was cultured in Brain Heart Infusion Broth (OXOID, UK) with added vancomycin (8 mg/ml). The BSI isolates were incubated at 37^{O}C in a shaking incubator for 13 to 18 hours in preparation for ASTs. Prior to AST testing 800 µl of each enriched BSI isolate was added to 40% Glycerol and stored in a -80°C cryo-freezer.

Antimicrobial Susceptibility Testing:

Antimicrobial Susceptibility testing was performed using the Kirby-Bauer disc diffusion method (Biemer, J.J., 1973, EUCAST guidelines). *Escherichia coli* (n=50) and Klebsiella (n=3) isolates were tested for susceptibility to ampicillin, cefotaxime, ceftazidime, imipenem, ertapenem, meropenem, gentamicin, ciprofloxacin, tigecycline, and trimethoprim using antimicrobial discs and for colistin susceptibility using the micro-broth dilution method. *Staphylococcus aureus* isolates (n=24) were tested for susceptibility to cefoxitin, linezolid and rifampicin using discs, and vancomycin using the micro-broth dilution method. *Enterococcus faecium* isolates (n=3) were tested for susceptibility to vancomycin, and linezolid using the disc diffusion assay. *Escherichia coli* ATCC 25922, *E. coli* NCTC 13846 (MCR-1 +ve), *E. faecalis* ATCC 29212, and *S. aureus*

ATCC 29213 strains were used as quality control strains. All ASTs were performed in accordance with EUCAST 2023/2024 guidelines.

DNA extraction and whole genome sequencing:

Microbial DNA was extracted from our isolates using the Macherey-Nagel nucleospin DNA isolation kit (Macherey-Nagel, CmbH Co.KG, Germany) according to the instructions provided. DNA concentrations were evaluated using a NanoDrop ™ spectrophotometer. Degradation quality checks were performed using gel electrophoresis in a 1% agarose gel (Duchefa Biochemie, The Netherlands). The extracted DNA was sent to Novogene Sequencing (Novogene (UK) Company Limited, Cambridge, UK) for short-read whole genome sequencing. The library preparation was performed on a Novseq X plus series illumina platform.

Genome assemblies and annotation:

The BSI isolate genomes were assembled and annotated using the following pipeline. All tools were used with default settings unless stated otherwise. Firstly, TrimGalore v0.6.10 (Krueger *et al.*, 2023) was used to trim and remove any adaptors from our sequences (with Cutadapt v0), they were then quality checked with FastQC v0.12.1(Krueger *et al.*, 2023). Hostile v.0.4.0 (Constantinides, Hunt and Crook, 2023) was run to remove any human contaminants. The BSI isolates were then assembled using Unicycler v0.4.8 (Wick *et al.*, 2017) with default paired-end settings. The genomes were then sequence-typed using MLST v2.23.0 (https://github.com/tseemann/mlst) and run against the PubMLST database (https://pubmlst.org/) to evaluate sequence clonal groups. Platon v1.7 (Schwengers *et al.*, 2020) was used to separate chromosomal and putative plasmid DNA. The genomes were then annotated using Prokka v1.11 (Seemann, 2014) and Bakta v1.91 (Schwengers *et al.*, 2021). Genomes were quality checked with CheckM v1.2.2 (Parks *et al.*, 2015) against the relevant species database. Completeness scores 90-95% were accepted for further analysis.

Evaluating Genomic Characteristics and Resistance Mechanisms

ABRicate v1.0.1(https://github.com/tseemann/abricate) was used to compare the BSI genome contigs against the following databases: The Comprehensive Antibiotic Resistance Database (CARD) (Jia *et al.*, 2017), Virulence Factor Database (VFDB) (Chen *et al.*, 2016), PlasmidFinder Database (PFDB) (Carattoli *et al.*, 2014), and the Antibacterial Biocide and Metal Resistance Genes Database (BacMet) (Pal *et al.*, 2014) bactranslated and with modified settings as described previously (Leigh *et al.*, 2022). ABRicate was run with an identity of 50-100%, allowing for full homolog detection and reduction of false negatives. R-studio (https://posit.co/download/rstudio-desktop/) was used to create a heatmap that visualized any antimicrobial resistant genes present within the isolates. The heatmap was created from running a script available at github (https://github.com/BioRRW/AMR-and-Virulence-factor-visualization-in-R/blob/main/Heatmap-Clustering.R#L99). The script was modified to fit our data. The heatmap was hierarchically clustered using the Ward. D method (Nielsen, 2016), isolates (columns) were clustered using the Euclidean distance method, while genes (rows) were clustered using the binary Jaccard distance method. Sankey Diagrams were created with Flourish (https://flourish.studio/) to compare replicon types between genomes. PointFinder (Zankari *et al.*, 2017) was used to identify point mutations. They were screened by querying the isolated genomes against the relevant species database within the PointFinder database. The *E. coli* genomes were separated by phylogroups using the *de novo* Clermontyper method (Beghain *et al.*, 2018). The tool, ezclermont v0.7.0 (https://github.com/nickp60/EzClermont), assigns phylogroups through a web-based application that utilizes assembled and annotated isolates. This tool has been modified from the original Clermont method (Clermont, Bonacorsi and Bingen, 2000). SCC*mec*Finder V.1.2 (Kondo *et al.*, 2007; Camacho *et al.*, 2009; 'Classification of Staphylococcal Cassette Chromosome *mec* (SCC *mec*): Guidelines for Reporting Novel SCC *mec* Elements', 2009) available at the Center for Genomic Epidemiology website (https://www.genomicepidemiology.org/) was used to evaluate the mobile genetic staphylococcal chromosomal cassette elements (SCC*mec*) within the MRSA genomes. Methicillin resistant *S. aureus* Spa typing was done using the Spa-typing service (Bartels *et al.*,

2014) available at the Center for Genomic Epidemiology website (https://www.genomicepidemiology.org/).

Pangenomic, Phylogenetic and Genomic clustering analysis:

The pangenome (the set of genes present across all the genomes within a species in this study) was constructed using Panaroo V1.4.1 (Tonkin-Hill *et al.*, 2020), with code available from Github (https://github.com/gtonkinhill/panaroo). Panaroo eliminates contamination and annotation errors originally associated with other pangenomic construction tools. Panaroo provides four different outputs, the "Core genes" (99% <= strains <= 100%), "Soft-Core genes" (95% <= strains <= 99%), "Shell-genes" (15% <= strains <= 95%), and "Cloud-genes" (0% <= strains <= 15%). The core genes were alignment trimmed with trimAL V1.4.rev15 build[2013-12-17] (Capella-Gutiérrez, Silla-Martínez and Gabaldón, 2009) and a maximum-likelihood phylogenetic tree was constructed with FastTree V2.1.11 (Price, Dehal and Arkin, 2009) with default settings . The tree was visualised using interactive tree of life (iTOL) (https://itol.embl.de/). Bootstrapping was used to provide statistical support to our tree with the score values as followed: >90% strongly supported, 70-90% well supported, 50-70% weakly supported, and <50% not supported (Baldauf, 2003). MASH V2.3 (Ondov *et al.*, 2016) was used to cluster and evaluate the similarity between genomes. MASH correlates with the average nucleotide identity (ANI). To measure the distance between genomes the k-mer size of 21 with a 10,000 MinHash sketch was used, in line with previous work (Abram *et al.*, 2021). Significant data with distances ≤0.05 and with a p-value ≤0.05 were reported in a Network analysis created with flourish (https://flourish.studio/).

RESULTS

Clinical Isolate Collection

Eighty BSI isolates were analyzed from three Irish hospitals (Hospital 1, Hospital 2, and Hospital 3). We received the most isolates from Hospital 3, followed by Hospital 2, and Hospital 1 (Table 1). The most abundant bacterial species across all hospitals were *E. coli* followed by *S. aureus* (Table 3.1).

Table 3.1: BSI isolates received from hospitals.

SPECIES	*HOSPITAL 1*	*HOSPITAL 2*	*HOSPITAL 3*	*TOTAL*
Escherichia coli	7	9	34	50
Klebsiella pneumoniae	-	1	1	2
Klebsiella aerogenes	-	-	1	1
Staphylococcus aureus	3	4	17	24
Enterococcus faecium	2	1	-	3

Antimicrobial Susceptibility Testing

For this study we requested ESBL and CP producing *E. coli* and *Klebsiella* BSI isolates, collected from patients in each hospital and identified as such by the hospital microbiology laboratory. All *E. coli* BSI (n = 50) were resistant to ampicillin (Figure 1). Resistance to cefotaxime varied across the hospitals from 100% to 56%. Ciprofloxacin resistance and trimethoprim resistances were detected in over 50% of isolates across all hospitals (Figure 1). All *E. coli* BSI isolates were susceptible to imipenem, ertapenem, meropenem, tigecycline and colistin. All *E. coli* received from each hospital were originally identified as ESBL producers, although after ASTs were performed some *E. coli* isolates were not resistant to either cephalosporin antimicrobials. One *E. coli* isolate was susceptible to all antimicrobials tested. There were no CP producing *E. coli* BSI collected from patients.

All *Klebsiella* (n = 3) isolates were resistant to ampicillin and were isolated from hospital 2 and 3 only. The Klebsiella Isolates were identified as ESBL (n =1) /CP (n = 2) producers by the hospital laboratories. Of those isolates, one *K. pneumoniae* and *K. aerogenes* were identified as CP producers while one other *K. pnuemoniae* was identified as an ESBL producer. The *Klebsiella* isolates received from hospital 2 were also resistant to cefotaxime, ceftazidime, and those from hospital 3 were both ertapenem resistant and one was also resistant to colistin. The *Klebsiella* isolates from hospital 3 (n = 2) were susceptible to imipenem, meropenem, cefotaxime, ceftazidime, gentamicin, and trimethoprim, while the isolate from hospital 2 was susceptible to imipenem, meropenem, gentamicin and trimethoprim.

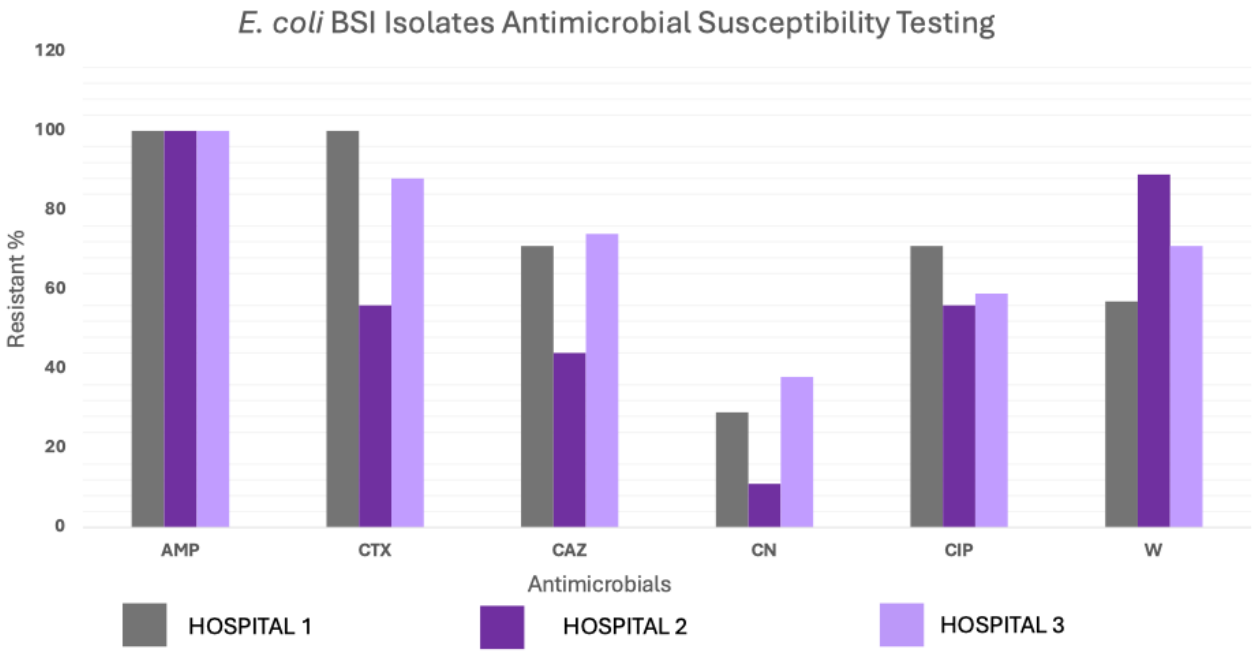


Figure 3.1: Antimicrobial Resistance Rates for *Escherichia coli* BSI. Each bar represents the overall percentage resistance per antibiotic from the total number of *E. coli* received per hospital. (AMP = Ampicillin, CTX = Cefotaxime, CAZ = Ceftazidime, CN = Gentamicin, CIP = Ciprofloxacin, W = Trimethoprim).

Oxacillin and cefoxitin weren't effective against *S. aureus* BSI isolates (n =24) received from hospitals 1, 2, and 3, with 100% resistance rates reported for all isolates (Figure 2). One *S. aureus* isolate from hospital 3 was also resistant to rifampicin. The *S. aureus* isolates from all hospitals were susceptible to vancomycin and linezolid. The three *E. faecium* isolates received from hospital 1 (n = 2) and hospital 2 (n = 1) were resistant to both vancomycin and ampicillin but were susceptible to linezolid.

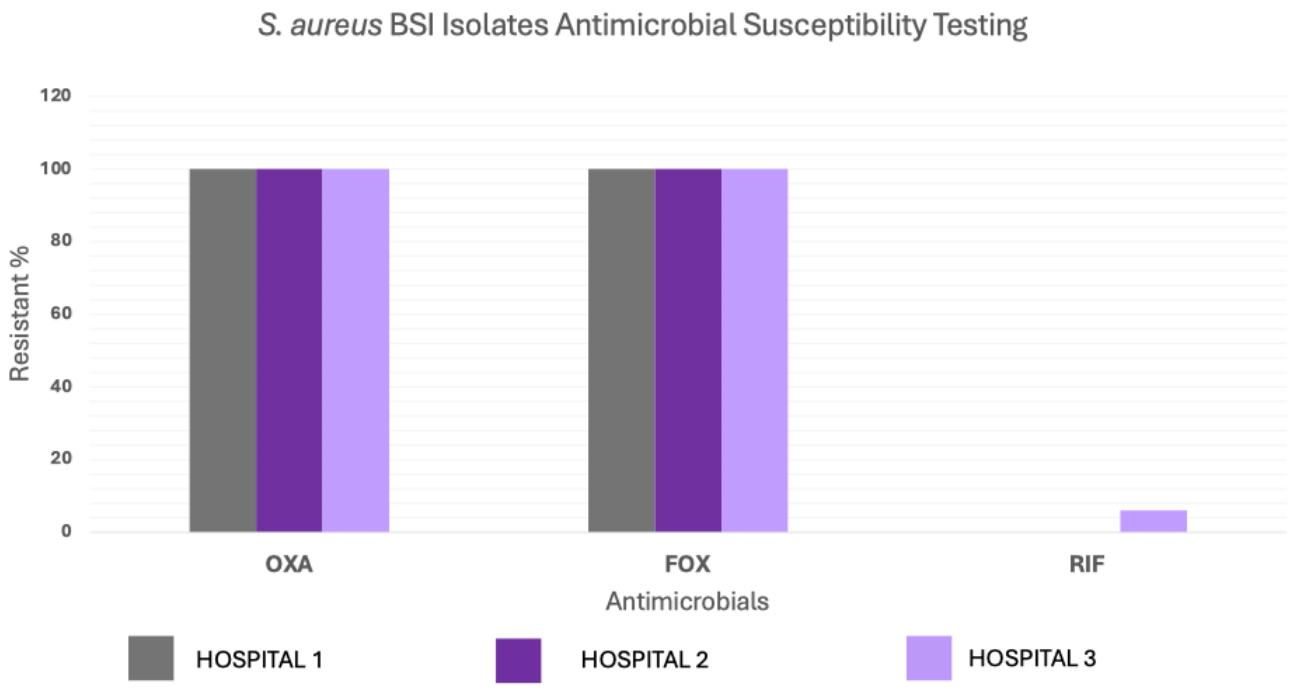


Figure 3.2: Antimicrobial Resistance Rates, *S. aureus* BSI isolates. Each bar represents the overall percentage resistance per antibiotic from the total number of *S. aureus* BSI isolates received per hospitals 1, 2, and 3. (OXA = Oxacillin, FOX = Cefoxitin, RIF = Rifampicin).

Phenotypical and Genotypical Characteristics present in the 80 BSI isolates.

All *E. coli* and *Klebsiella* isolates received from each hospital were suspected ESBL-producers, phenotypically some of these isolates were confirmed non-ESBL producers. Isolates phenotypically resistant to three or more antimicrobial classes were classified as multi-drug resistant. The multi-locus sequence typing (MLST) of the *E. coli* BSI isolates separated them into 18 distinct sequence types (STs) that corresponded to 10 different clonal complexes (Table 3.2). Of the sequence types reported ST131 were the most prevalent (n = 32) followed by ST38 (n = 4), and ST69 (n =3). *Escherichia coli* ST131 were frequent throughout hospital 1 (n = 5 of 7 isolated), hospital 2 (n = 6 of 9 isolated) and hospital 3 (n = 21 of 34 isolated). Eleven *E. coli* sequence types (ST12, ST121, ST469, ST998, ST59, ST642, ST14, ST73, ST23, ST95, ST362) were reported once from isolates received from all three hospitals. *Escherichia coli* ST998, ST642, and ST362 were not assigned to a clonal complex.

Surface antigen proteins (O, H, K) were investigated to assign the O:H serotypes. Three serotypes were associated with the *E. coli* ST131 clones: Onovel31:H4, O16:H5, and O153var1:H5 (Table 3.2). All *E. coli* ST131 clones were assigned to phylogroup B2. Most *E. coli* ST131 Onovel31:H4 clones harboured the ESBL, $bla_{CTX\text{-}M\text{-}15}$ gene, across all three hospitals, while the 3 remaining *E. coli* ST131 Onovel31:H4 clones harboured the $bla_{CTX\text{-}M\text{-}27}$ gene from Hospital 2 only. The $bla_{CTX\text{-}M\text{-}27}$ gene appeared in *E. coli* ST131 O16:H5 from hospitals 1 and 3.

Escherichia coli ST38 were present in Hospital 3 only, and were spilt between two different serotypes –O153var1:H30 and O15:H18, all four isolates were assigned to phylogroup D. *Escherichia coli* ST38 O153var1:H30 all harboured the $bla_{CTX\text{-}M\text{-}9}$ gene, while *E. coli* ST38 O15:H18 harboured the $bla_{CTX\text{-}M\text{-}15}$ gene. Also assigned to phylogroup D were the *E. coli* ST69 isolates with serotypes O15:H18 and O17:H18, two *E. coli* ST69 isolates were present in Hospital 3 and one in Hospital 1. The ST69- O17:H18 isolates present in Hospital 3 harboured $bla_{CTX\text{-}M\text{-}15}$ while ST69-O15:H18 from Hospital 1 harboured $bla_{CTX\text{-}M\text{-}27}$. Interestingly, $bla_{CTX\text{-}M\text{-}9}$ was only detected in isolates from Hospital 3.

Table 3.2: Phenotypic and genotypic characteristics of *E. coli* BSI isolates. Date refers to the date of collection of the isolate from a patient. Abbreviations: ST (sequence type), ARG # (The total number of antimicrobial resistant genes per isolate), MDR (The isolate is multi-drug resistant - resistant to three or more antimicrobial classes), ESBL-P (isolate produces Extended-Spectrum Beta-lactamase enzymes), ESBL Gene (s) (Extended-Spectrum-Beta-Lactamase genes present within each isolate.

ID	HOSPITAL	DATE	ST	CLONAL CPLX	SEROTYPE	PHYLOGROUP	RESISTANCE PHENOTYPE	ARG #	MDR	ESBL-P	ESBL GENE(S)
EC1 (3)	3	23/04/2023	ST121	ST121 CPLX	O18:H5	B2	CTX, CAZ, AMP, CN	54		ESBL-P	*blaCTX-M-15*
EC2 (3)	3	15/05/2023	ST469	ST469 CPLX	O9:H9	B1	AMP, CN, W, CIP	60	MDR		
EC3 (3)	3	16/05/2023	ST131	ST131 CPLX	Onovel31:H4	B2	CTX, CAZ, AMP, CN, W, CIP	62	MDR	ESBL-P	*blaCTX-M-15*
EC4 (3)	3	19/05/2023	ST38	ST38 CPLX	O153var1:H30	D	CTX, CAZ, AMP, W	64		ESBL-P	*blaCTX-M-9*
EC5 (3)	3	22/05/2023	ST69	ST69 CPLX	O15:H18	D	CTX, AMP, W	58		ESBL-P	*blaCTX-M-15*
EC6 (3)	3	31/05/2023	ST998	UNASSIGNED	O2:H7	B2	CTX, AMP, CN, W	61	MDR	ESBL-P	*blaCTX-M-27*
EC7 (3)	3	09/06/2023	ST131	ST131 CPLX	Onovel31:H4	B2	CTX, CAZ, AMP, CIP	56		ESBL-P	*blaCTX-M-15*
EC8 (3)	3	20/06/2023	ST131	ST131 CPLX	Onovel31:H4	B2	CTX, CAZ, AMP, CN, W, CIP	65	MDR	ESBL-P	*blaCTX-M-15*
EC9 (3)	3	28/06/2023	ST131	ST131 CPLX	Onovel31:H4	B2	CTX, CAZ, AMP, W, CIP	60	MDR	ESBL-P	*blaCTX-M-15*
EC10 (3)	3	03/05/2023	ST131	ST131 CPLX	Onovel31:H4	B2	CTX, CAZ, AMP, W, CIP	60	MDR	ESBL-P	*blaCTX-M-15*
EC11 (3)	3	03/05/2023	ST14	ST14 CPLX	O18:H5	B2	AMP, CN, W, CIP	57	MDR		
EC12 (3)	3	20/07/2023	ST95	ST95 CPLX	O18:H7	B2	CTX, CAZ, AMP	53		ESBL-P	*blaCTX-M-15*
EC13 (3)	3	09/08/2023	ST69	ST69 CPLX	O17:H18	D	CTX, AMP	52		ESBL-P	*blaCTX-M-15*
EC14 (3)	3	04/09/2023	ST131	ST131 CPLX	Onovel31:H4	B2	CTX, CAZ, AMP, CIP	59		ESBL-P	*blaCTX-M-15*
EC15 (3)	3	06/09/2023	ST131	ST131 CPLX	Onovel31:H4	B2	CTX, CAZ, AMP, CN, CIP	55	MDR	ESBL-P	*blaCTX-M-15*
EC16 (3)	3	18/09/2023	ST23	ST23 CPLX	O8:H4	C	AMP, CN, W, CIP	60	MDR		
EC17 (3)	3	24/09/2023	ST131	ST131 CPLX	Onovel31:H4	B2	CTX, CAZ, AMP, CN, W, CIP	61	MDR	ESBL-P	*blaCTX-M-15*
EC18 (3)	3	24/10/2023	ST131	ST131 CPLX	Onovel31:H4	B2	CTX, CAZ, AMP, CN, W, CIP	60	MDR	ESBL-P	*blaCTX-M-15*
EC19 (3)	3	29/10/2023	ST38	ST38 CPLX	O15:H18	D	CTX, CAZ, AMP, CN, W, CIP	63	MDR	ESBL-P	*blaCTX-M-15*
EC20 (3)	3	18/11/2023	ST131	ST131 CPLX	O153var1:H5	B2	CTX, CAZ, AMP	60		ESBL-P	*blaCTX-M-15*
EC21 (3)	3	18/11/2023	ST131	ST131 CPLX	Onovel31:H4	B2	CTX, CAZ, AMP, W, CIP	59	MDR	ESBL-P	*blaCTX-M-15*

ID	HOSPITAL	DATE	ST	CLONAL CPLX	SEROTYPE	PHYLOGROUP	RESISTANCE PHENOTYPE	ARG #	MDR	ESBL-P	ESBL GENE(S)
EC22 (3)	3	25/11/2023	ST38	ST38 CPLX	O153var1:H30	D	CTX, CAZ, AMP, W, CIP	64	MDR	ESBL-P	*blaCTX-M-9*
EC23 (3)	3	25/01/2024	ST131	ST131 CPLX	O16:H5	B2	CTX, AMP, W, CIP	62	MDR	ESBL-P	*blaCTX-M-15*
EC24 (3)	3	01/02/2024	ST131	ST131 CPLX	Onovel31:H4	B2	CTX, CAZ, AMP, W, CIP	59	MDR	ESBL-P	*blaCTX-M-15*
EC25 (3)	3	22/02/2024	ST131	ST131 CPLX	Onovel31:H4	B2	CTX, CAZ, AMP, CN, W, CIP	60	MDR	ESBL-P	*blaCTX-M-15*
EC26 (3)	3	04/03/2024	ST131	ST131 CPLX	O16:H5	B2	CTX, CAZ, AMP, W	54		ESBL-P	*blaCTX-M-27*
EC27 (3)	3	10/03/2024	ST131	ST131 CPLX	Onovel31:H4	B2	CTX, CAZ, AMP, W, CIP	57	MDR	ESBL-P	*blaCTX-M-15*
EC28 (3)	3	27/03/2024	ST131	ST131 CPLX	O16:H5	B2	CTX, CAZ, AMP, W, CIP	57	MDR	ESBL-P	*blaCTX-M-15*
EC29 (3)	3	12/04/2024	ST131	ST131 CPLX	Onovel31:H4	B2	CTX, CAZ, AMP	56		ESBL-P	*blaCTX-M-15*
EC30 (3)	3	16/04/2024	ST73	ST73 CPLX	O22:H1	B2	NONE	51			
EC31 (3)	3	28/04/2024	ST131	ST131 CPLX	Onovel31:H4	B2	CTX, CAZ, AMP, W, CIP	60	MDR	ESBL-P	*blaCTX-M-15*
EC32 (3)	3	29/04/2024	ST38	ST38 CPLX	O153var1:H30	D	CTX, CAZ, AMP, W, CIP	64	MDR	ESBL-P	*blaCTX-M-9*
EC33 (3)	3	30/04/2024	ST131	ST131 CPLX	Onovel31:H4	B2	CTX, AMP, W	58		ESBL-P	*blaCTX-M-15*
EC34 (3)	3	09/05/2024	ST131	ST131 CPLX	Onovel31:H4	B2	CTX, CAZ, AMP, CN, W, CIP	60	MDR	ESBL-P	*blaCTX-M-15*
EC1 (2)	2	21/04/2023	ST131	ST131 CPLX	Onovel31:H4	B2	AMP, CN, W	58	MDR		
EC2 (2)	2	24/04/2023	ST1642	UNASSIGNED	O8:H7	B1	AMP, W, CIP	59	MDR		
EC3 (2)	2	03/05/2023	ST12	ST12 CPLX	O4:H5	B2	AMP, W	57		ESBL-P	*blaSHV-102*
EC4 (2)	2	13/07/2023	ST362	UNASSIGNED	O11:H9	D	CTX, AMP, W	58			
EC5 (2)	2	n/a	ST131	ST131 CPLX	Onovel31:H4	B2	AMP, W, CIP	62		ESBL-P	*blaCTX-M-27*
EC6 (2)	2	16/10/2023	ST131	ST131 CPLX	O16:H5	B2	CTX, CAZ, AMP	54		ESBL-P	*blaCTX-M-15*
EC7 (2)	2	04/08/2023	ST131	ST131 CPLX	Onovel31:H4	B2	CTX, CAZ, AMP, W, CIP	62	MDR	ESBL-P	*blaCTX-M-27*
EC8 (2)	2	14/08/2023	ST131	ST131 CPLX	Onovel31:H4	B2	CTX, CAZ, AMP, W, CIP	59	MDR	ESBL-P	*blaCTX-M-27*
EC9 (2)	2	16/07/2023	ST131	ST131 CPLX	Onovel31:H4	B2	CTX, CAZ, AMP, W, CIP	58	MDR	ESBL-P	*blaCTX-M-15*

ID	HOSPITAL	DATE	ST	CLONAL CPLX	SEROTYPE	PHYLOGROUP	RESISTANCE PHENOTYPE	ARG #	MDR	ESBL-P	ESBL GENE(S)
EC1 (1)	1	19/11/2023	ST131	ST131 CPLX	Onovel31:H4	B2	CTX, CAZ, AMP, CIP	54		ESBL-P	*blaCTX-M-15*
EC2 (1)	1	24/11/2023	ST131	ST131 CPLX	O16:H5	B2	CTX, AMP, CN, W	63	MDR	ESBL-P	*blaCTX-M-27*
EC3 (1)	1	03/12/2023	ST59	ST59 CPLX	O1:H7	F	CTX, CAZ, AMP, CIP	53		ESBL-P	*blaCTX-M-3*
EC4 (1)	1	04/12/2023	ST131	ST131 CPLX	Onovel31:H4	B2	CTX, AMP, W	58		ESBL-P	*blaCTX-M-15*
EC5 (1)	1	18/12/2023	ST131	ST131 CPLX	O16:H5	B2	CTX, CAZ, AMP, W, CIP	62	MDR	ESBL-P	*blaCTX-M-27*
EC6 (1)	1	20/01/2024	ST69	ST69 CPLX	O17:H18	D	CTX, CAZ, AMP, CN, W, CIP	56	MDR	ESBL-P	*blaCTX-M-27*
EC7 (1)	1	31/01/2024	ST131	ST131 CPLX	Onovel31:H4	B2	CTX, CAZ, AMP, CN, CIP	55	MDR	ESBL-P	*blaCTX-M-15*

Within the three *Klebsiella* isolates collected two were identified as *Klebsiella pneumoniae* and one *Klebsiella aerogenes* (Table 3.3). The two *Klebsiella* isolates collected from patients in Hospital 3 carried the carbapenemase gene bla_{OXA-48}. Both *K. pneumoniae* displayed different sequence types, with *K. pneumoniae* ST39 isolated in Hospital 3 and *K. pneumoniae* ST5275-1LV isolated in Hospital 2. *Klebsiella pneumoniae* ST5275-1LV carried both $bla_{CTX-M-15}$ and $bla_{SHV-110}$ genes. *Klebsiella aerogenes* displayed the sequence type ST404. This isolate was also phenotypically resistant to colistin (MIC: 8 μg/ml). At present, no member of the mobile colistin resistance (*mcr*) gene family was identified within this isolate. All three *Klebsiella* isolates were susceptible to imipenem, meropenem, and ciprofloxacin antimicrobials. *Klebsiella aerogenes* ST404 and *K. pneumoniae* ST39 were further susceptible to cefotaxime and ceftazidime antimicrobials.

Table 3.3: Phenotypic and genotypic characteristics of *Klebsiella* BSI isolates. Abbreviations: ST (sequence type), ARG # (The total number of antimicrobial resistant genes per isolate, ESBL-P (isolate produces Extended-Spectrum Beta-lactamase enzymes), ESBL Gene (s) (Extended-Spectrum-Beta-Lactamase genes present within each isolate), CPE Genes (Carbapenemase enzymes resistant genes present within each isolate.

ID	HOSPITAL	DATE	ST	RESISTANT PHENOTYPE	ARG #	ESBL GENES	CPE GENES
KP1 (3)	3	08/04/2023	ST39	ETP, AMP	43		*blaOXA-48*
KA1 (3)	3	08/08/2023	ST404	ETP, AMP, COL	44		*blaOXA-48*
KP1 (2)	2	18/01/2024	ST5275-1LV	CTX, CAZ, AMP	45	*blaCTX-M-15*, *blaSHV-110*	

Sequence type ST22 was the predominant sequence type within the 24 *S. aureus* BSI isolates (n = 12) (Table 3.4), and they were also all clonal complex 22 (CC22). Most of the ST22 isolates (n = 9) were collected from Hospital 3 while two were collected from Hospital 2. Staphylococcal cassette chromosomal (SCC*mec*) complexes were investigated for all *S. aureus*. The SCCmec_type_IV(2B) predominated across the *S. aureus* regardless of ST. However, all ST22 isolates were also SCCmec_type_IV(2B). The ST5 was detected across all hospitals but no SCCmec element could be assigned to these isolates. ST5 isolates were of the spa type t311 and t9676. ST5-t311 isolates commonly contain SCC*mec*-Type-II- complexes but this element could not be found within our isolates. ST5 isolates were collected from Hospital's 3 (n = 3) and 1 (n = 2). ST92-V isolates were collected from Hospital 3 only, and ST1-IVa/IV isolates were collected from Hospital's 2 and 1. All isolates contained the *mecA* gene associated with MRSA as only MRSA were included in this study.

Table 3.4: Phenotypical and genotypical characteristics of *S. aureus* BSI isolates. Abbreviations: ST (sequence type), SCC*mec* Element (*Staphylococcal* cassette chromosome *mec* type), ARG # (The total number of antimicrobial resistant genes per isolate.

ID	HOSPITAL	DATE	ST	Clonal CPLX	SCCmec ELEMENT	spa TYPE	RESISTANT PHENOTYPE	ARG #	mec GENE
MRSA1 (3)	3	23/04/2023	ST5	CC5	n/a	t311	FOX, RIF, OXA	16	*mecA*
MRSA2 (3)	3	22/05/2023	ST22	CC22	SCCmec_type_IV(2B)	t22	FOX, OXA	16	*mecA*
MRSA3 (3)	3	08/06/2023	ST22	CC22	SCCmec_type_IVj(2B)	n/a	FOX, OXA	13	*mecA*
MRSA4 (3)	3	14/06/2023	ST8	CC8	SCCmec_type_IVa(2B)	t8	FOX, OXA	12	*mecA*
MRSA5 (3)	3	12/07/2023	ST22	CC22	SCCmec_type_IVj(2B)	n/a	FOX, OXA	12	*mecA*
MRSA6 (3)	3	26/07/2023	ST5	CC5	n/a	t9676	FOX, OXA	15	*mecA*
MRSA7 (3)	3	03/09/2023	ST22	CC22	SCCmec_type_IVb(2B)	n/a	FOX, OXA	12	*mecA*
MRSA8 (3)	3	08/04/2023	ST22	CC22	SCCmec_type_IV(2B)	t22	FOX, OXA	12	*mecA*
MRSA9 (3)	3	09/11/2023	ST97	CC97	SCCmec_type_V(5C2)	n/a	FOX, OXA	12	*mecA*
MRSA10 (3)	3	19/09/2023	ST5	CC5	n/a	t311	FOX, OXA	16	*mecA*
MRSA11 (3)	3	27/09/2023	ST97	CC97	SCCmec_type_V(5C2)	t12805	FOX, OXA	12	*mecA*
MRSA12 (3)	3	11/01/2023	ST22	CC22	SCCmec_type_IV(2B)	t22	FOX, OXA	13	*mecA*
MRSA13 (3)	3	27/01/2023	ST97	CC97	SCCmec_type_V(5C2)	n/a	FOX, OXA	11	*mecA*
MRSA14 (3)	3	16/03/2023	ST22	CC22	SCCmec_type_IV(2B)	t32	FOX, OXA	13	*mecA*
MRSA15 (3)	3	14/04/2023	ST22	CC22	SCCmec_type_IV(2B)	t32	FOX, OXA	13	*mecA*
MRSA16 (3)	3	05/01/2024	ST22	CC22	SCCmec_type_IV(2B)	t32	FOX, OXA	12	*mecA*
MRSA17 (3)	3	05/03/2024	ST22	CC22	SCCmec_type_IVc(2B)	t19	FOX, OXA	13	*mecA*
MRSA1 (2)	2	23/06/2023	ST22	CC22	SCCmec_type_IV(2B)	t32	FOX, OXA	12	*mecA*
MRSA2 (2)	2	22/04/2023	ST22	CC22	SCCmec_type_IV(2B)	t32	FOX, OXA	12	*mecA*
MRSA3 (2)	2	27/02/2024	ST1	CC1	SCCmec_type_IVa(2B)	t127	FOX, OXA	17	*mecA*
MRSA4 (2)	2	01/09/2024	ST1	CC45	SCCmec_type_IV(2B)	t230	FOX, OXA	13	*mecA*
MRSA1 (1)	1	25/11/2023	ST5	CC5	n/a	t311	FOX, OXA	16	*mecA*
MRSA2 (1)	1	27/12/2023	ST1	CC1	SCCmec_type_IVa(2B)	t127	FOX, OXA	17	*mecA*
MRSA3 (1)	1	07/01/2024	ST5	CC5	n/a	t311	FOX, OXA	15	*mecA*

Enterococcus faecium isolates were identified as ST80/ clonal complex 17 (ST80-CC17) (Table 3.5). Of the three isolates collected two were from Hospital 1, and one was from Hospital 2. All three isolates were resistant to ampicillin and vancomycin. Each isolate was susceptible to linezolid. All isolates contained the *vanA* gene cluster with *vanHA, vanRA, vanXA, vanYA* and *vanZA,* while VRE2 from hospital 1 contained an additional *vanSA* gene.

Table 3.5: Phenotypic and Genotypic characteristics of *E. faecium* BSI isolates. Abbreviations: ST (sequence type), CLONAL CPLX (clonal complex), ARG # (The total number of antimicrobial resistant genes per isolate).

ID	HOSPITAL	DATE	ST	CLONAL CPLX	RESISTANT PHENOTYPE	ARG #	*van* gene
VRE1 (2)	2	11/02/2023	ST80	CC17	AMP, VAN	17	*vanA, vanHA, vanRA, vanXA, vanYA, vanZA*
VRE1 (1)	1	25/12/2023	ST80	CC17	AMP, VAN	15	*vanA*, *vanHA*, *vanRA*, *vanXA*, *vanYA*, *vanZA*.
VRE 2 (1)	1	24/01/2024	ST80	CC17	AMP, VAN	17	*vanA, vanHA, vanRA, vanSA, vanXA, vanYA, vanZA*

Resistance Mechanisms: Antimicrobial Resistance Genes, Metal/ Biocide Resistance Genes, Virulence Factors, Point Mutations.

Antimicrobial Resistance Genes

On average there were 58 AMR genes present across all *E. coli* BSI isolates 44 presents across all *Klebsiella* isolates, 14 presents across all *S. aureus* isolates, and 16 presents across all *E. faecium* isolates. Many AMR genes were located on the chromosomes of isolates including the *mecA* complex (n = 24/ 100%) in *S. aureus* isolates herein known as MRSA isolates (Table 3.3). Some ESBL genes - $bla_{CTX\text{-}M\text{-}27}$ and $bla_{CTX\text{-}M\text{-}09}$ – were located on the chromosomes of *E. coli* isolates. Several AMR genes were present on plasmid contigs (figure 3.3). ESBL genes ($bla_{CTX\text{-}M\text{-}15}$ (n =19 /66%), $bla_{CTX\text{-}M\text{-}3}$ (n = 1 / 100%), were present on plasmid contigs, in addition to the *vanA* operons present on plasmid contigs within *E. faecium* isolates herein known as VREfm isolates (Table 3.4). Carbapenem resistant *K. aerogenes* and *K. pneumoniae* contained one plasmid orientated AMR gene – $bla_{OXA\text{-}48}$.

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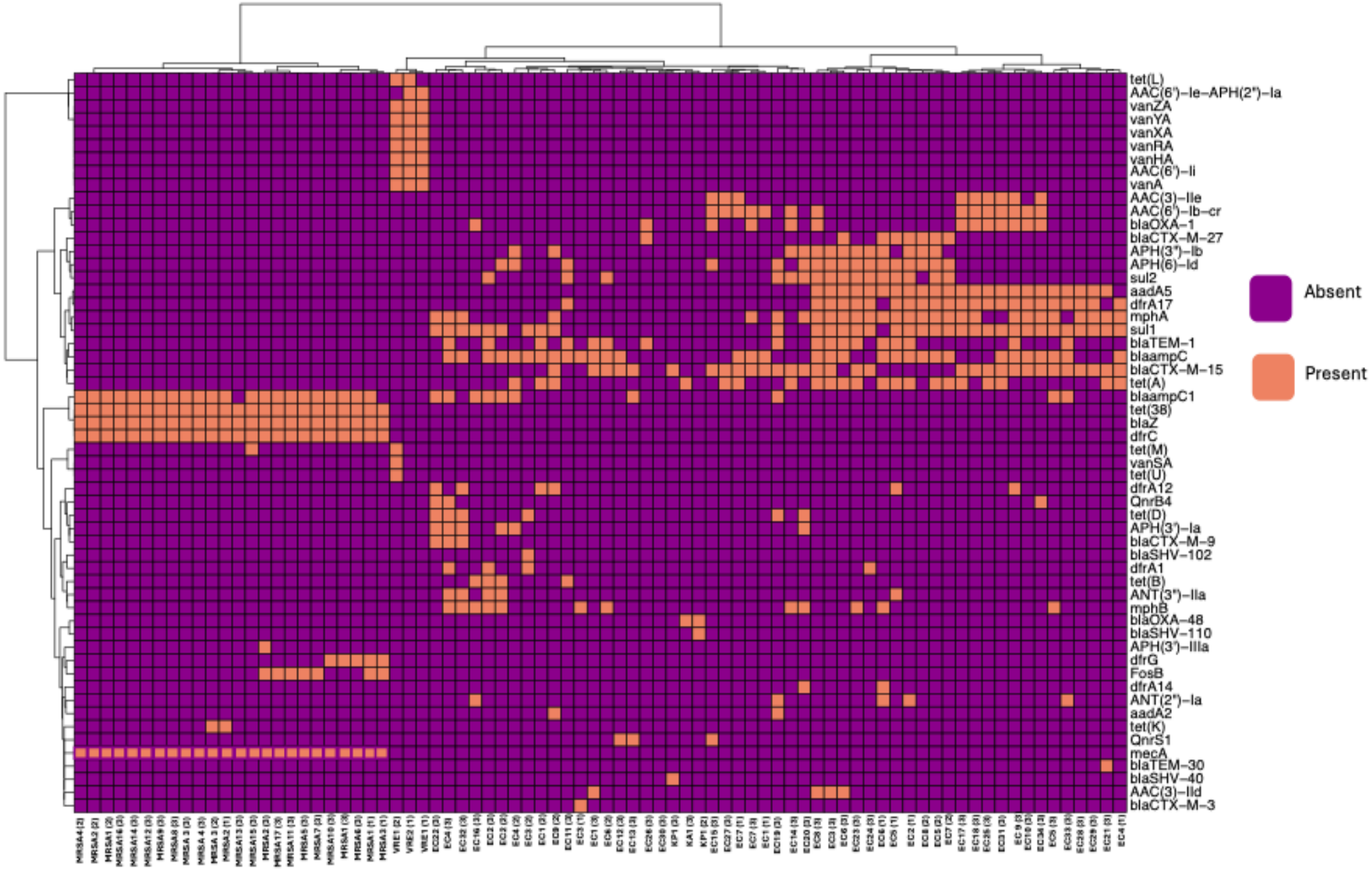

tet(L)
AAC(6')−Ie−APH(2")−Ia
vanZA
vanYA
vanXA
vanRA
vanHA
AAC(6')−Ii
vanA
AAC(3)−IIe
AAC(6')−Ib−cr
blaOXA−1
blaCTX−M−27
APH(3")−Ib
APH(6)−Id
sul2
aadA5
dfrA17
mphA
sul1
blaTEM−1
blaampC
blaCTX−M−15
tet(A)
blaampC1
tet(38)
blaZ
dfrC
tet(M)
vanSA
tet(U)
dfrA12
QnrB4
tet(D)
APH(3')−Ia
blaCTX−M−9
blaSHV−102
dfrA1
tet(B)
ANT(3")−IIa
mphB
blaOXA−48
blaSHV−110
APH(3')−IIIa
dfrG
FosB
dfrA14
ANT(2")−Ia
aadA2
tet(K)
QnrS1
mecA
blaTEM−30
blaSHV−40
AAC(3)−IId
blaCTX−M−3
Absent
Present

Figure 3.3: Hierarchically clustered heatmap displaying some antimicrobial resistance genes present/absent throughout 80 BSI isolates. These genes were selected as they were not ubiquitous across the genomes. The Isolates with (3) are from Hospital 3, while isolates with (2) are from Hospital 2, and (1) are from Hospital 1. Columns were clustered using Euclidean distance for isolates, while the rows (genes) were clustered using Jaccard distance to compare the present/absent of each gene within each isolate. Wards method was used to hierarchically cluster the heatmap. Purple boxes represent the gene being absent and orange boxes represent the gene being present.

Virulence Factors.

On average there were 116 virulence factors present on the chromosomes across all the *E. coli* BSI isolates and 2 on plasmid contigs within the *E. coli* isolates. For the *Klebsiella* isolates there were an average of 53 present on the chromosomes of all isolates and 1 present within plasmid contigs. For MRSA isolates there were no virulence factors present on plasmid contigs but an average of 68 were present on across all MRSA chromosomes. There was an average of 14 virulence factors reported across the chromosomes of VRE isolates and 1 was present on plasmid contigs.

The most common toxins, adhesins, protectins, evasions, and siderophores are summarized in table 3.6a and table 3.6b. Of the toxins, *senB*, which encodes the shiga-toxin, ShET-2, was reported in 48% (n = 24) of *E. coli* isolates and one of the *Klebsiella* isolates. Interestingly *senB* co-occurred with other toxin encoding genes, *sat* (Sat) and *astA* (EAST1) in 8% (n = 4) of *E. coli* isolates. The gene encoding the autotransporter toxin Sat was alone in 30% of isolates (n =15) and present with s*enB* in 38% (n =19) of *E. coli* isolates. Some isolates contained zero toxins, including two *E. coli* ST131 isolates. However, all bacteria were isolated from bloodstream infections. One *K. pneumoniae and one K. aerogenes* contained *astA* and both *senB* and *astA,* respectively. *K. pneumoniae* ST5275-1LV from hospital 2 contained no toxins. The most common toxins present in MRSA co-occurred – *seb, selq, selk* – in 41% (n = 10) of isolates. There were no toxins reported for VREfm isolates.

Most adhesins present in the *E. coli* isolates included: *papG* (58%), *fdeC* (88%), *yagW/ecpD* (80%), and *fimH* (14%). The y*agW/ecpD* virulence factor was further identified alone in all three *Klebsiella* isolates. The *icaA-D* adhesin operon was present in 100% (n = 24) of MRSA isolates with an added *clfB* gene which encodes for the clumping factor B fibrinogen-binding protein was present in 71% (n = 17) of isolates. VREfm contained adhesins *acm* (100% / n = 3) and *scm* (33% / n = 1).

Virulence factors that encode proteins with invasive/evasive functions against host cells comprise protectins or evasions and siderophores or leukocidins (Table 6b). Protectins such as *ompA*, *entF/E* and *icsP/sopA* were reported in all *E. coli* (n = 50) and *Klebsiella* isolates (n = 3). Genes such as *aur, hlgA-C, hly/hla,* and *hid/hib* were detected in all MRSA isolates (n =24), *luk-PV* was further reported in 46% (n = 11) of MRSA. Genes that encode siderophores were reported in *E. coli* isolates with *ybtE* and *iucC* occurring together in 62% (n = 31) of isolates. The *iuc* gene cluster was reported in 12% of isolates (n = 6).

Table 3.6a: Prevalence of Virulence factors throughout Gram- negative BSI isolates. This table is separated by *E. coli* and *Klebsiella* isolates. ST – sequence type.

ID	ST-SEROTYPE	PHYLOGROUP	TOXIN	ADHESINS	PROTECTINS	SIDEROPHORES
				***Escherichia* Isolates**		
EC1 (3)	ST121-O18:H5	B2	senB	FocH, fdeC, papG, yagW/ecpD	icsP/sopA, entF, entE, ompA	ybtE
EC2 (3)	ST469-O9:H9	B1	n/a	fdeC, fimH, yagW/ecpD	icsP/sopA, entF, entD, entE, ompA	ybtE
EC3 (3)	ST131-Onovel31:H4	B2	sat, senB	fdeC, papG, yagW/ecpD	icsP/sopA, entF, entE, ompA	ybtE, iucC
EC4 (3)	ST38-O152var1H:30	D	sat	fdeC, fimH, papG, yagW/ecpD	ompA, entF, entE, shuV	iucA, iucB, iucC, ybtE
EC5 (3)	ST69-O15:H18	D	n/a	fdeC, fimH, papG, yagW/ecpD	ompA, entF, entE, shuV	ybtE
EC6 (3)	ST998-O2:H7	B2	senB	fdeC, sfaH, yagW/ecpD	icsP/sopA, entF, entE, ompA	ybtE
EC7 (3)	ST131-Onovel31:H4	B2	sat	fdeC, papG, yagW/ecpD	icsP/sopA, entF, entE, ompA	ybtE, iucC
EC8 (3)	ST131-Onovel31:H4	B2	sat, senB	fdeC, papG, yagW/ecpD	icsP/sopA, entF, entE, ompA	ybtE, iucC
EC9 (3)	ST131-Onovel31:H4	B2	astA, sat, senB	fdeC, papG, yagW/ecpD	icsP/sopA, entF, entE, ompA	ybtE, iucC
EC10 (3)	ST131-Onovel31:H4	B2	sat	fdeC, papG, yagW/ecpD	icsP/sopA, ompA	iucC
EC11 (3)	ST14-O18:H5	B2	senB	fdeC, yagW/ecpD	icsP/sopA, entF, entE, ompA	ybtE, iucC
EC12 (3)	ST95-O18:H7	B2	n/a	fdeC, sfaH, yagW/ecpD	ompA, entF, entE	ybtE
EC13 (3)	ST69-O17:H18	D	sat	fdeC, papG, yagW/ecpD	ompA, entF, entE, shuV	iucA, iucB, iucC,
EC14 (3)	ST131-Onovel31:H4	B2	sat, senB	fdeC, draP, afaD, afaC-I, afaB-I, afaA, yagW/ecpD	ompA, entF, entE	ybtE
EC15 (3)	ST131-Onovel31:H4	B2	sat	fdeC, papG, yagW/ecpD	ompA, entF, entE	ybtE, iucC
EC16 (3)	ST23-O8:H4	C	n/a	fdeC, fimH, afaE-VIII, afaD-VIII, afaC-VIII, afaB-VIII, afaA-VIII,	ompA, entF, entE	ybtE, iucC
EC17 (3)	ST131-Onovel31:H4	B2	sat, senB	fdeC, papG, yagW/ecpD	ompA, entF, entE	iucC, ybtE

ID	ST-Serotype	Phylogroup	Toxin	Adhesins	Protectins	Siderophores
EC18 (3)	ST131-Onovel31:H4	B2	sat	fdeC, papG, yagW/ecpD	ompA, entF, entE, shuV	iucC, ybtE
EC19 (3)	ST38-O15:H18	D	sat, senB	yagW/ecpD, fimH, draC, draP, afaE-II, afaA, daaF	ompA, entF, entE	iucC, ybtE
EC20 (3)	ST131-O153var1:H5	B2	sat	fdeC, draE2, draP, afaD, afaC-I, afaB-I, draA, yagW/ecpD	ompA, entF, entE	iucC, ybtE
EC21 (3)	ST131-Onovel31:H4	B2	sat	fdeC, draE2, draP, afaD, afaC-I, afaB-I, afaA, yagW/ecpD	ompA, entF, entE, shuV	ybtE
EC22 (3)	ST38-O152var1H:30	D	sat	fimH, papG, yagW/ecpD	ompA, entF, entE	ybtE, iucC, iucA iucB
EC23 (3)	ST131-O16:H5	B2	sat, senB	fdeC, draE2, draP, afaD, afaC-I, afaB-I, afaA, yagW/ecpD	ompA, entF, entE	ybtE, iucC
EC24 (3)	ST131-Onovel31:H4	B2	sat, senB	fdeC, yagW/ecpD, afaA, afaB-I, afaC-I, afaD, draP, afaE-I,	ompA, entF, entE	ybtE, iucC
EC25 (3)	ST131-Onovel31:H4	B2	sat, senB	fdeC, papG, yagW/ecpD	ompA, entF, entE	ybtE, iucC
EC26 (3)	ST131-O16:H5	B2	sat, senB	fdeC, papG, yagW/ecpD	ompA, entF, entE	ybtE, iucC
EC27 (3)	ST131-Onovel31:H4	B2	astA, sat, senB	fdeC, papG, yagW/ecpD	ompA, entF, entE	ybtE, iucC
EC28 (3)	ST131-O16:H5	B2	senB	fdeC, papG, yagW/ecpD	ompA, entF, entE	ybtE
EC29 (3)	ST131-Onovel31:H4	B2	sat	fdeC, yagW/ecpD, sfaH	ompA, entF, entE	ybtE, iucC
EC30 (3)	ST73-O22:H1	B2	n/a	fdeC, yagW/ecpD, sfaH	ompA, entF, entE	ybtE
EC31 (3)	ST131-Onovel31:H4	B2	sat, senB	fdeC, papG, yagW/ecpD	ompA, entF, entE, icsP/sopA	ybtE, iucC
EC32 (3)	ST38-O152var1H:30	D	sat	fdeC, fimH, papG, yagW/ecpD	ompA, entF, entE, shuV	ybtE, iucC, iucA iucB
EC33 (3)	ST131-Onovel31:H4	B2	sat, senB	fdeC, papG, yagW/ecpD	ompA, entF, entE, icsP/sopA	ybtE, iucC
EC34 (3)	ST131-Onovel31:H4	B2	sat	fdeC, papG, yagW/ecpD	ompA, entF, entE, icsP/sopA	ybtE, iucC
EC1 (2)	ST131-Onovel31:H4	B2	sat, senB	afaA, afaB-I, afaC-I, afaD, afaE-I, draP, fdeC, yagW/ecpD	icsP/sopA, entF, entE, ompA	ybtE, iucC

ID	ST-Serotype	Phylogroup	Toxin	Adhesins	Protectins	Siderophores
EC2 (2)	ST1642-O8:H7	B1	n/a	fdeC, yagW/ecpD	ompA, entF, entE, entD	iucA, iucB, iucC,
EC3 (2)	ST12-O4:H5	B2	senB	FocH, yagW/ecpD	icsP/sopA, entF, entE, ompA	ybtE
EC4 (2)	ST362-O11:H9	D	n/a	fdeC, fimH, yagW/ecpD	ompA, entF, entE, shuV	iucA, iucB, iucC, ybtE
EC5 (2)	ST131-Onovel31:H4	B2	astA, sat, senB	fdeC, papG, yagW/ecpD	icsP/sopA, entF, entE, ompA	ybtE, iucC
EC6 (2)	ST131-O16:H5	B2	senB	fdeC, draE2, draP, afaD, afaC-I, afaB-I, afaA, yagW/ecpD		ybtE
EC7 (2)	ST131-Onovel31:H4	B2	astA, sat, senB	fdeC, papG, yagW/ecpD	ompA, entF, entE,	ybtE, iucC
EC8 (2)	ST131-Onovel31:H4	B2	n/a	fdeC, yagW/ecpD	ompA, entF, entE	n/a
EC9 (2)	ST131-Onovel31:H4	B2	sat	fdeC, papG, yagW/ecpD	ompA, entF, entE	ybtE, iucC
EC1 (1)	ST131-Onovel31:H4	B2	sat	fdeC, papG, yagW/ecpD	entF, entE, ompA	ybtE, iucC
EC2 (1)	ST131-O16:H5	B2	sat, senB	fdeC, papG, yagW/ecpD	entF, entE, ompA	ybtE, iucC
EC3 (1)	ST59-O1:H7	F	sat, senB	fimH, fdeC, papG	entD, entF, entE, ompA	ybtE
EC4 (1)	ST131-Onovel31:H4	B2	n/a	fdeC, draE2, draP, afaD, afaC-I, afaB-I, afaA, yagW/ecpD	n/a	ybtE
EC5 (1)	ST131-O16:H5	B2	senB	fedC, yagW/ecpD	entF, entE, ompA	ybtE
EC6 (1)	ST69-O17:H18	D	sat, senB	fdeC, fimH, papG, yagW/ecpD	entF, entE, ompA	ybtE, iucC
EC7 (1)	ST131-Onovel31:H4	B2	sat	fdeC, papG, yagW/ecpD	entF,entE, shuV	ybtE, iucC
				***Klebsiella* Isolates**		
KP1 (3)	ST39	n/a	astA	yagW/ecpD	ompA, entE, entF	n/a
KA1 (3)	ST404	n/a	astA, senB	yagW/ecpD	ompA, entE, entF	n/a
KP1 (2)	ST5275-1LV	n/a	n/a	yagW/ecpD	ompA, entE, entF	n/a

Table 3.6b: Prevalence of Virulence factors throughout Gram- positive BSI isolates. The table is separated by MRSA and VREfm isolates.

ID	ST	Phylogroup	Toxins and Evasions	Adhesins	Leukocidin Genes
			MRSA		
MRSA1 (3)	ST5	n/a	seb, selq, selk, aur, hlgB, hlgC, hlgA, hld, hlb, hly/hla	icaC, icaB, icaD, icaA, clfB	lukF-PV
MRSA2 (3)	ST22	n/a	sec, sell, seb, selk, selq, aur, hlgB, hlgC, hlgA, hld, hlb, hly/hla	icaC, icaB, icaD, icaA, clfB	n/a
MRSA3 (3)	ST22	n/a	selq, selk, seb, aur, hlgB, hlgC, hlgA, hld, hlb, hly/hla	icaC, icaB, icaD, icaA, clfB	n/a
MRSA4 (3)	ST8	n/a	selk, aur, hlgB, hlgC, hlgA, hld, hlb,	icaC, icaB, icaD, icaA, clfB	lukF-PV
MRSA5 (3)	ST22	n/a	selq, selk, seb, aur, hlgB, hlgC, hlgA, hld, hly/hla	icaC, icaB, icaD, icaA, clfB	n/a
MRSA6 (3)	ST5	n/a	selq, selk, seb, aur, hlgB, hlgC, hlgA, hld, hlb, hly/hla	icaC, icaB, icaD, icaA, clfB	lukF-PV
MRSA7 (3)	ST22	n/a	selq, selk, seb, aur, hlgB, hlgC, hlgA, hld, hlb, hly/hla	icaC, icaB, icaD, icaA, clfB	n/a
MRSA8 (3)	ST22	n/a	selq, selk, seb, aur, hlgB, hlgC, hlgA, hld, hlb, hly/hla	icaC, icaB, icaD, icaA,	n/a
MRSA9 (3)	ST97	n/a	aur, hlgB, hlgC, hlgA, hld, hlb, hly/hla	icaC, icaB, icaD, icaA, clfB	lukF-PV
MRSA10 (3)	ST5	n/a	seb, selq, selk, aur, hlgB, hlgC, hlgA, hld, hlb, hly/hla	icaC, icaB, icaD, icaA, clfB	lukF-PV
MRSA11 (3)	ST97	n/a	aur, hlgB, hlgC, hlgA, hld, hlb, hly/hla	icaC, icaB, icaD, icaA, clfB	lukF-PV
MRSA12 (3)	ST22	n/a	seb, selq, selk, aur, hlgB, hlgC, hlgA, hld, hlb, hly/hla	icaC, icaB, icaD, icaA,	n/a
MRSA13 (3)	ST97	n/a	aur, hlgB, hlgC, hlgA, hld, hlb, hly/hla	icaC, icaB, icaD, icaA, clfB	lukF-PV
MRSA14 (3)	ST22	n/a	sec, sell, seb, selk, selq, aur, hlgB, hlgC, hlgA, hld, hlb, hly/hla	icaC, icaB, icaD, icaA,	n/a
MRSA15 (3)	ST22	n/a	sec, sell, seb, selk, selq, aur, hlgB, hlgC, hlgA, hld, hlb, hly/hla	icaC, icaB, icaD, icaA,	n/a
MRSA16 (3)	ST22	n/a	seb, selq, selk, sell, aur, hlgB, hlgC, hlgA, hld, hlb, hly/hla	icaC, icaB, icaD, icaA,	n/a
MRSA17 (3)	ST22	n/a	selk, seb, aur, hlgB, hlgC, hlgA, hld, hlb, hly/hla	icaC, icaB, icaD, icaA, clfB	n/a
MRSA1 (2)	ST22	n/a	selq, selk, seb, aur, hlgB, hlgC, hlgA, hld, hlb, hly/hla	icaC, icaB, icaD, icaA, clfB	n/a
MRSA2 (2)	ST22	n/a	selq, selk, seb, aur, hlgB, hlgC, hlgA, hld, hlb, hly/hla	icaC, icaB, icaD, icaA,	n/a
MRSA3 (2)	ST1	n/a	seh, aur, hlgB, hlgC, hlgA, hld, hlb,	icaC, icaB, icaD, icaA,	lukF-PV
MRSA4 (2)	ST1	n/a	sell, sec, seb, selk, sel, qaur, hlgB, hlgC, hlgA, hld, hlb,hly/hla	icaC, icaB, icaD, icaA, clfB	n/a
MRSA1 (1)	ST5	n/a	sea, seb, selk, selq, aur, hlgB, hlgC, hlgA, hld, hlb,	icaC, icaB, icaD, icaA, clfB	lukF-PV
MRSA2 (1)	ST1	n/a	aur, hlgB, hlgC, hlgA, hld, hlb,	icaC, icaB, icaD, icaA, clfB	lukF-PV

ID	ST	Phylogroup	Toxins and Evasions	Adhesins	Leukocidin Genes
MRSA3 (1)	ST5	n/a	selk, selq, aur, hlgB, hlgC, hlgA, hld, hlb,	icaC, icaB, icaD, icaA, clfB	lukF-PV
			VREfm		
VRE1 (2)	ST80	n/a	n/a	acm,	n/a
VRE1 (1)	ST80	n/a	n/a	acm, scm	n/a
VRE2 (1)	ST80	n/a	n/a	acm,	n/a

Metal Resistance Genes

There was an average of 146 MRGs identified on the chromosomes of *E. coli* BSI isolates. An average of 1 MRG was present across *E. coli* plasmid contigs. *Klebsiella* isolates reported an average of 128 MRGs on their chromosomes with 23 MRGs reported across *K. pneumoniae* plasmid contigs. There was an average of 13 MRGs on MRSA chromosomes and an average of 1 MRG per plasmid contig. VRE reported an average of 8 MRGs per chromosome and 0 per plasmid contig.

Within the *E. coli* BSI isolates the gene *qacEdelta1*, conferring resistance to quaternary ammonium compounds, was reported in over 50% of isolates. An operon of genes conferring mercury resistance (*merA*/C/D/E/P/R1/T) were reported in 12% of isolates, and the gene *kpnO* – causing resistance to multiple biocides such as peroxides and biguanides- was reported 4% of isolates. Genes: *qacEdelta*, *merA* gene cluster, and *KpnO*, were the only genes reported within *E. coli* plasmid contigs. There were high levels of *copA* (90%) and *cusA/ybdE* (92%) which cause copper (*copA, cusA*) and silver (*ybdE*) resistance reported in addition to *mar A* (92%) which cause cyclohexane, phenyl, and alkane resistance, and *baeR/S* (86%) genes which result in zinc, sodium deoxycholate (SDC), and tungsten resistance, within the chromosomes of the *E. coli* isolates. Some other MRGs of interest included *G2alt* (aluminum resistance / n = 6 / 12 %), *gadA* (hydrochloric acid resistance / n = 27 / 54%), *arsA* (arsenic resistance / n = 21 / 42%), and *acrR/ybaH* (acriflavine resistance / n= 25 / 50%).

Of the *Klebsiella* isolates*, K. pneumoniae* ST39 from hospital 3 contained 23 MRGS within plasmid contigs conferring resistance to arsenic (*ars* genes), iron (*fec* genes), copper (*pco* genes), and silver (*sil* genes). Like *E. coli* isolates, genes conferring acriflavine, zinc, sodium deoxycholate (SDC), tungsten, and aluminum resistance were detected on chromosome contigs of all three *Klebsiella* isolates.

Plasmid contigs from the MRSA contained MRGs such as *cadD*, which confers resistance to cadmium and zinc and was reported in 46% of isolates, *mco* – copper and cobalt resistance- reported in 13% of isolates, *copB* – copper and silver- reported in 13% of

isolates and *ars* gene clusters, which confers resistance to arsenic. *CopA* was detected within chromosome contigs for 33% of isolates while other MRGS such as *nixA* – nickel resistance- was detected in 75% of isolates and *sh-fabl* – triclosan resistance-, sodA – peroxide resistance-, and *G2alt* were present in all isolates. There were no MRGs within VREfm plasmid contigs, however VREfm isolates contained *cop* gene clusters, *perR* – conferring peroxide resistance -, *chtS* – biguanides resistance -, and *sodA/B* genes within chromosome contigs of all isolates.

Point mutations.

Single nucleotide mutations leading to amino acid changes were evaluated in each of the BSI isolates by comparing the genomes with the Pointfinder database. *E. coli* isolates were found to have mutations within genes: *gyrA*, *parC,* and *parE,* conferring resistance to fluroquinolones, including ciprofloxacin. Of the genes, *gyrA* mutations dominated within 76% of *E. coli* isolates, followed by *parE* mutations (66%), and *parC* mutations (62%).

Mutations in the genes: *acrR*, *ompK36*, and *ompK37* were identified in the *Klebsiella* isolates. The single amino acid change in the *acrR* gene results in ciprofloxacin resistance, while single amino acid changes in *ompK36* and *ompK37* resulted in carbapenem resistance. *Klebsiella pneumoniae* ST39 and *K. aerogenes* from hospital 3 both carry the bla_{OXA-48} gene and within *K. pneumoniae* ST39 the *ompK37* gene has two single amino acid changes – isoleucine to methionine- at separate positions- I128M, I70M- leading to carbapenem resistance. *K. aerogenes* has one single acid change within the *ompK36* gene at position A217S changing alanine to serine and leading to carbapenem resistance. Interestingly, both the above isolates are phenotypically resistant to ertapenem, however *K. pneumoniae* ST5275-1LV from hospital 2 has the same mutations at the same positions within both the *ompK36* and *ompK37* genes that led to carbapenem resistance and in the absence of the bla_{OXA-48} gene, this isolate is not resistant to imipenem, ertapenem or meropenem.

MRSA isolates reported mutations in genes: *grlA* (38%), *grlB* (21%), *gyrA* (33%) which all confer ciprofloxacin resistance. Single amino acid mutations were further reported in

fusA (25%), *dfrB* (4%) and *ileS* (4%), resulting in fusidic acid, trimethoprim, and mupirocin resistance.

VRE isolates reported the most mutations within the *pbp5* gene resulting in ampicillin resistance in all isolates. All VRE isolates contained further mutations in *gyrA* and *parC*, which report resistance to ciprofloxacin.

Plasmid Replicon Types.

E. coli and *Klebsiella* BSI isolates contained plasmid contigs with over 20 different replicon types (Figure 5). Two main replicon types were reported throughout all three hospitals – Incompatibility (Inc) type plasmids and Colicin (Col) type plasmids. Of the two replicon types the Inc-type plasmids dominated and at least one Inc-type plasmid was detected in each *E. coli* and Klebsiella isolates. *E. coli* ST131-Onovel 31:H4 isolates contained the most replicon types (n = 19) with Inc-type plasmids reported in 68% of the isolates and Col-type plasmids reported in 32%. IncFIB (AP001918) _1 was most frequently detected in the *E. coli* isolates (n = 41 / 82%) and present across all hospitals. The IncFIB replicon co-occurred with IncFIA_1 in 47% of isolates from hospital 3, 44% of isolates from hospital 2, and 14% of isolates from hospital 1. IncFIB (AP001918) _1 co-occurred with Col156_1 in 47% of isolates from hospital 3, 67% of isolates from hospital 2, and 71% of isolates from hospital 1. IncFIB (AP001918) _1 was not present in *Klebsiella* isolates; however, IncL/M(p-OXA-48) was reported within carbapenem resistant *K. aerogenes* ST404 and *K. pneumoniae* ST39 isolates collected from hospital 3. Within *K. pneumoniae* ST39 the IncL/M(p-OXA-48) replicon type co-occurred with IncFIB(K)_1. *K. pneumoniae* ST5275-1LV from hospital 2 contained four different replicon types – Col44011_1 (this replicon type was further reported within *E. coli* ST131/ ST73 isolates from hospital 2), Col4401_1, IncFIA_1 (reported within *E. coli* isolates from Hospitals 3 and 1), and InFII_1. Replicon type IncN_1 was reported in ST131-Onovel:31:H4 only, it is present in one isolate from hospital 3. IncI2_1_Delta was reported only once in ST362-O11:H9 which is an isolate collected from hospital 2. IncX1_1, IncX3_1, IncX4_1, and IncX8_ are present in one ST131-Onovel31:H4 isolate from hospital 3.

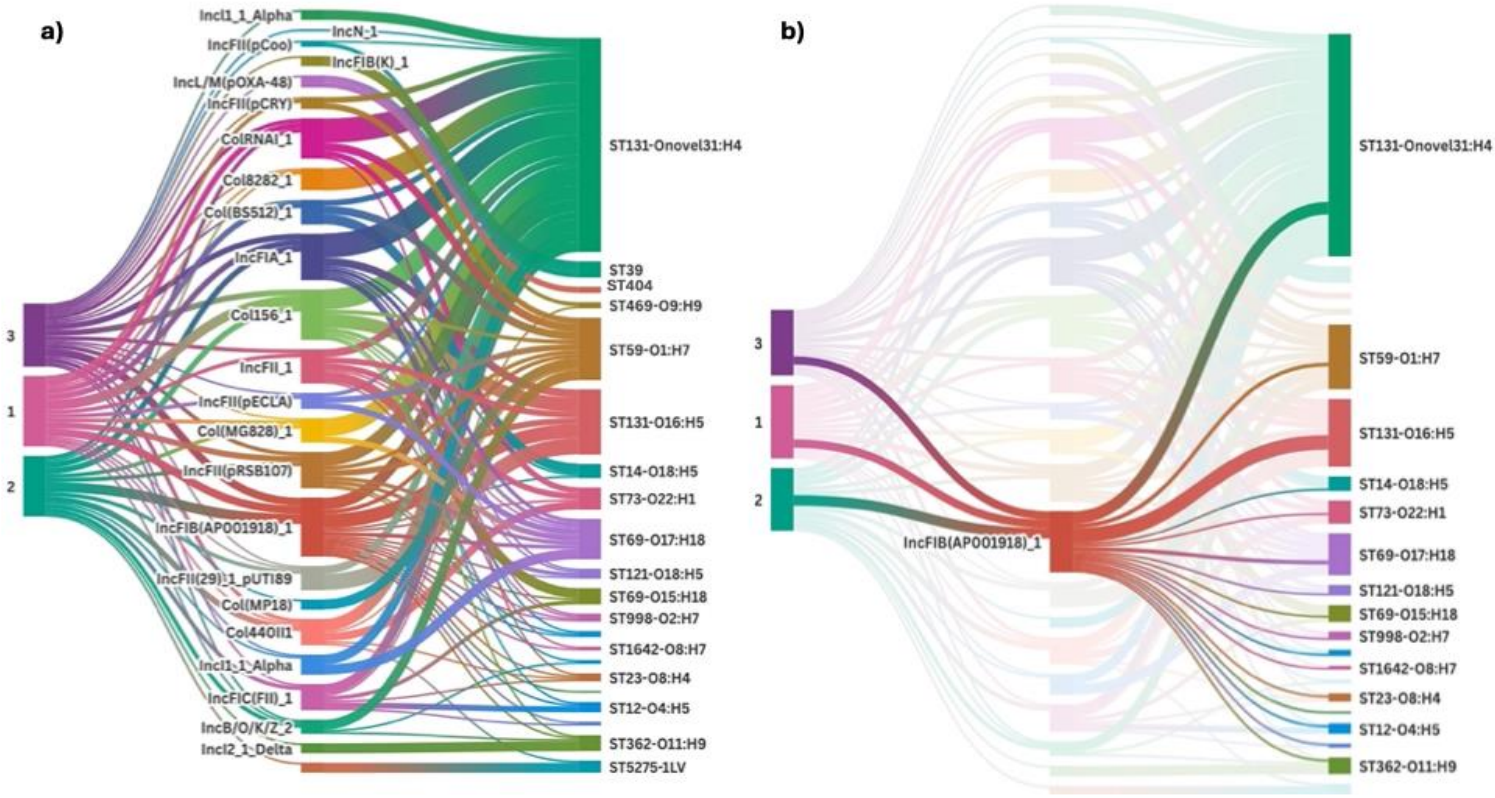
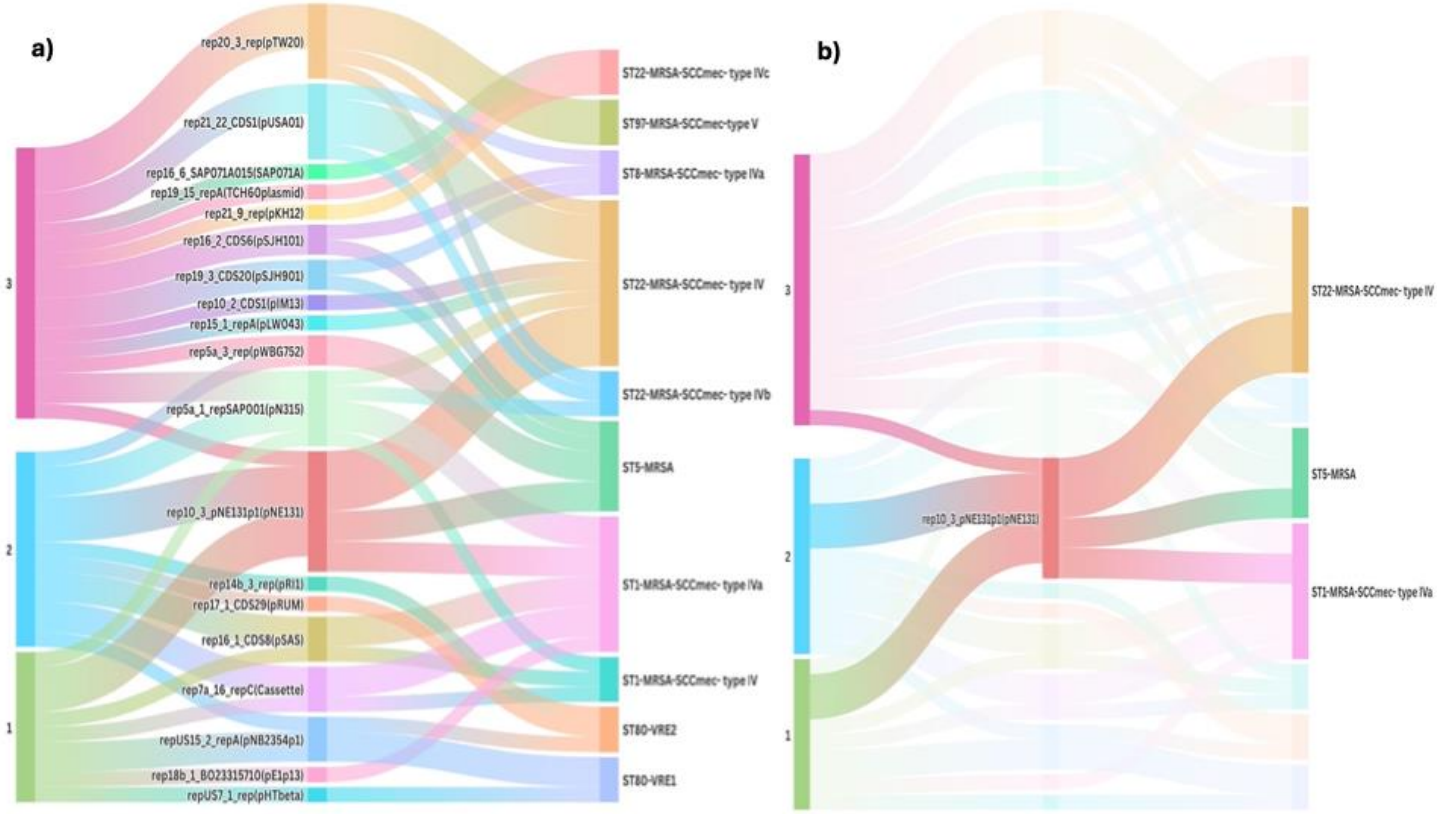


Figure 3.5: a) Sankey diagram displaying plasmid replicon types occurring within *E. coli* and *Klebsiella* isolates per hospital's 3, 2, and 1. The bars on the left represent the three hospitals, the bars in the middle display the different replicon types and the bars on the right are the isolates, which are grouped by sequence type/serotypes. Replicon types with % identity $\geq 80\%$ were included. b) Sankey diagram displaying the most detected replicon type from the *E. coli* isolates, compared to the total background.

Six MRSA isolates did not contain plasmid contigs and of the isolates that did, 16 different replicon types were detected. Of the MRSA replicon types, Rep5a_1_repSAP001(pN315) and Rep10_3_pNE131p1(pNE131) were detected across all hospitals. Four replicon types were detected across hospitals 1 and 2, while MRSA from hospital 3 contained replicon types (n =9) unique to isolates from hospital 3 only. Rep10_3_pNE131p1(pNE131) was the most frequently detected replicon (n = 8 / 44%) and detected across all hospitals. VRE plasmid contigs contained five different replicon

types within the three isolates. VRE ST-80 isolates contained the replicon type - RepUS15_2_repA(pNB2354p1) which was present in VRE isolates collected from hospital 1 and 2. Rep14b_3_rep(pRI1) and Rep17_1_CDS29(pRUM) were detected in both ST1-MRSA-SCC*mec* type IVa and ST 80 VRE collected from hospital 2.

Figure 3.6: a) Sankey diagram displaying the plasmid replicon types occurring within



MRSA and VRE isolates per hospital. The bars on the left represent the three hospitals, the bars in the middle display the different replicon types and the bars on the right are the isolates, which are grouped by sequence type/serotypes. Replicon types with % identity $\geq 80\%$ were include. b) Sankey diagram displaying the replicon type which reported most frequently in the MRSA and VRE isolates, compared to the total background.

Comparative Genomic Relatedness via MASH Clustering Networks.

The *E. coli* chromosomal genomes cluster together within their sequence type and are more genetically related, than isolates outside their cluster (Figure 6a). The largest

cluster represents the *E. coli* ST131 – Onovel31:H4 isolates which were the most abundant clone isolated (Figure 6a). ST131-Onovel31:H4 clones from each of the three hospitals cluster together indicating close relatedness between their chromosomal genomes independent to their location. Interestingly, other *E. coli* ST131 clones do not cluster with Onovel:H4 isolates indicating differences within their chromosomal genetic makeup. Single dots represent *E. coli* isolates assigned eleven different sequence types that share the least genomic similarity with each of the clusters and each other. Single dots not linked to another dot are genetically unique.

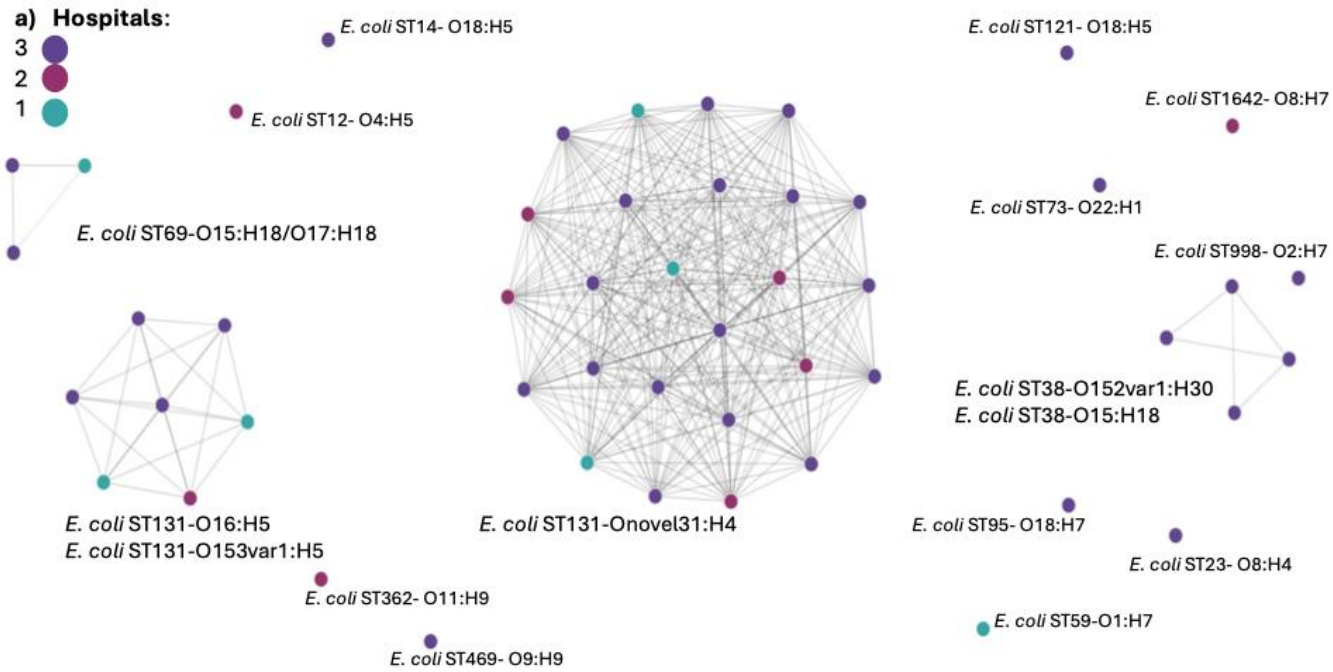


Figure 3.6: Network graph displaying *E. coli* chromosomal genome clustering via MASH distancing. Genome distances ≤0.05 with a P-value ≤0.05 were reported. Dots colored purple represent isolates collected from hospital 3, pink represent isolates collected from hospital 2, and blue dots represent isolates collected from hospital 1. Isolates clustered closely together are the most genetically similar – there are smaller distances between their genomes. The largest network in the middle of the figure represents *E. coli*

ST131-Onovel31:H4 isolates. The seven-isolate cluster on the bottom left represent *E. coli* ST131-O16:H5 and *E. coli* – O152var1:H5 isolates. The three-isolate cluster on the top left represent *E. coli* ST69-O15:H18/O17:H18 isolates. The four-isolate cluster on the bottom right represents *E. coli* ST38 isolates. The single dots represent eleven disconnected sequence types (DSS).

The plasmid contigs within the *E. coli* isolates share more similarity than their chromosomal genome (Figure 6b). The majority of *E. coli* plasmid contigs from all three hospitals cluster together within a large network. The isolates closer to the center are more genetically related than those around the edge. All *E. coli* ST131 clones except two from hospital 1 and 3, cluster within the larger network. Interestingly, there are two *E. coli* ST131-Onovel31:H4 isolates which do not share the replicon type – IncFIB (AP001918) _1 and they do not cluster within the larger network, instead they are represented by the single purple and blue dots shown on the bottom right of figure 6 b). Of the other four isolates not included within the major cluster, IncFIB (AP001918) _1 is not present within them, however there were two isolates within the cluster that did not contain IncFIB (AP001918) _1.

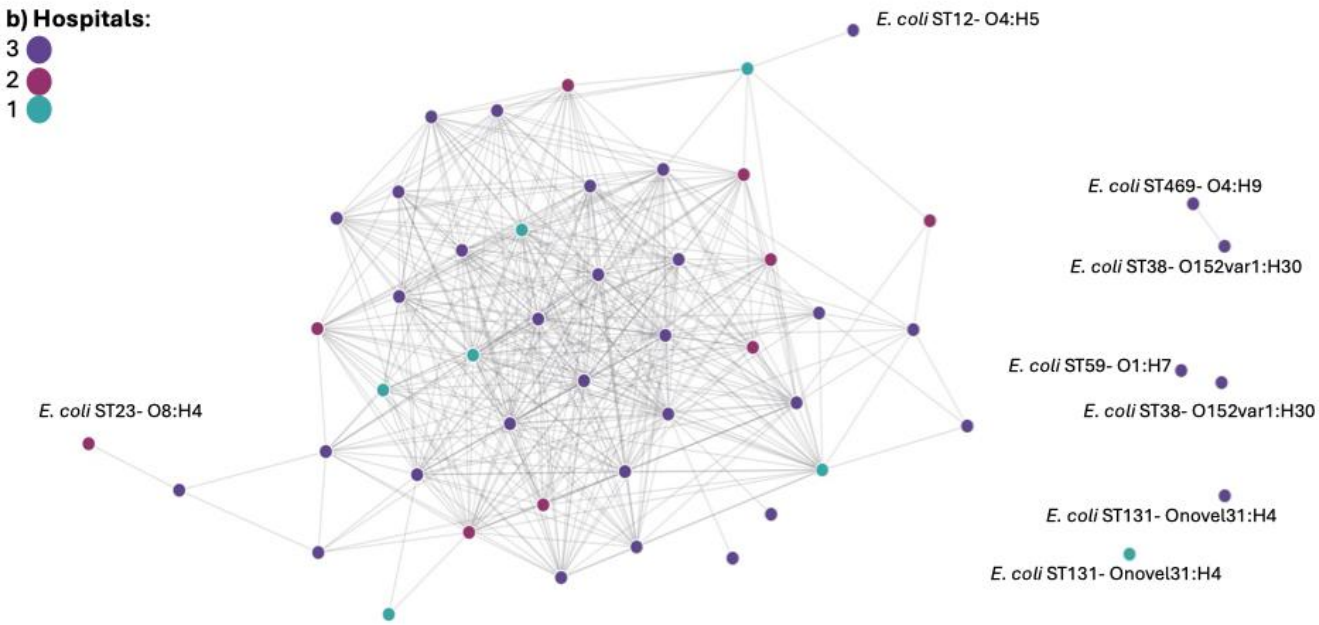


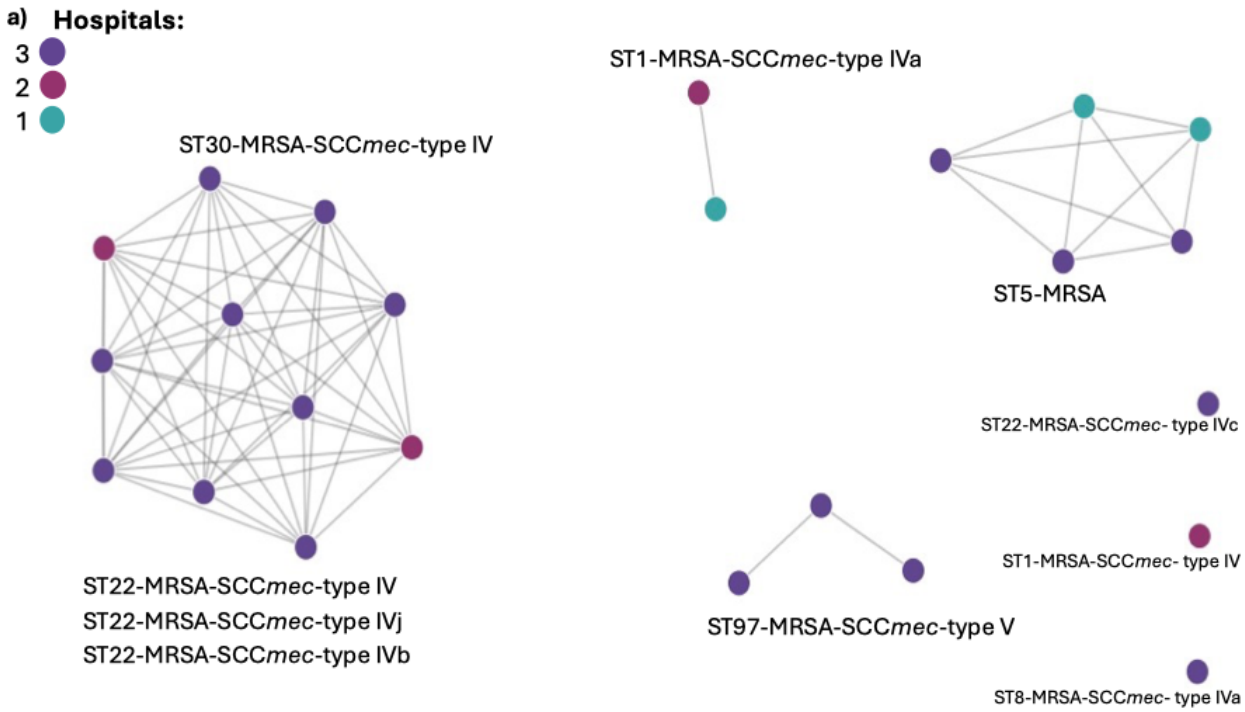
Figure 3.7: Network graph displaying *E. coli* replicon-type clustering via MASH distancing. Genome distances ≤0.05 with a P-value ≤0.05 were reported. Dots colored purple represent isolates collected from hospital 3, pink represent isolates collected from hospital 2, and blue dots represent isolates collected from hospital 1. Isolates clustered closely together are the most genetically similar – there are smaller distances between their genomes.

Klebsiella pneumoniae ST5275-1LV from hospital 2 and *K. pneumoniae* ST39 from hospital 3 share a genome distance of 0.008 in both their plasmid and chromosomal genomes, indicating high genetic similarities, but they are not genetically identical.

MRSA isolates cluster according to SCC*mec* type, ST22 MRSA isolates from type IV cluster together and are more genetically related than ST22-MRSA-SCC*mec* type IVc which is represented on figure 7 a), by the first single purple dot on the right side of the figure. The same can be seen with ST1-MRSA-SCCmec type IVa which connect (both collected from separate hospitals) but do not connect to ST1-MRSA-SCC*mec* type IV which is represented by the pink single dot on the right side of the figure. MRSA isolates

collected from each of the three hospitals are genetically similar, with two isolates (ST22-MRSA type IV) collected from hospital 2- at different collection dates- possibly identical. These possibly identical isolates share a MASH distance value of 0, p-value 0 and 10000/10000 Mash Shares. These two isolates are present in the large cluster with the same sequence type: ST22-MRSA-SCCmec- type IV.

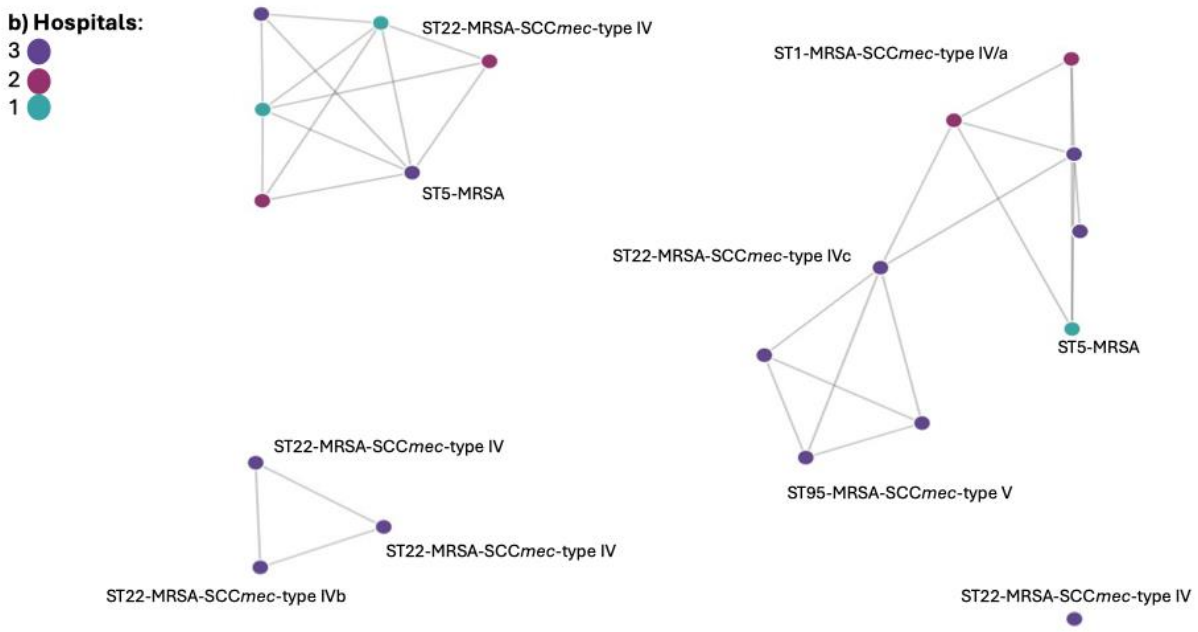
Figure 3.8: a) Network graph displaying MRSA chromosomal relatedness via MASH Distancing. Colored dots represent isolates collected from each of the three hospitals, which can be seen in the figure legend. The three colored dots on the bottom right represent disconnected sequence types (DSS), they do not cluster with any other isolate.



MRSA plasmid contigs were genetically similar between isolates collected from each of the three hospitals, as seen from the clusters in figure 7 b). Isolates did not cluster as tightly as their chromosomal counterparts, possibly indicating more differences between their plasmid genomes. One isolate represented by the single purple dot (name of isolate

here) does not cluster with any other, interestingly it is the only isolate to contain the replicon type, rep15_1_repA(pLW043).

Figure 3.9: b) Network graph displaying MRSA replicon type relatedness via MASH Distancing. All twenty-four isolates were searched against the PlasmidFinder database and there were no plasmid replicon types reported in six isolates. Thus, the nineteen isolates with plasmid replicon types reported where analyzed via MASH Distancing.



VRE isolates collected from hospital 1 were more genomic similar than the isolates collected from hospital 2 in both plasmid and chromosomal genomes. VRE plasmid contigs between hospital isolates were less like their chromosomal genetic makeup. Isolates that are identical report a mash distance of 0 and mash shares 10000/10000, as can be seen by the three rows in table 7. The first three rows report isolates VRE1 (1) ST80, VRE2 (1) ST80 and VRE1 (2) ST80 searched against themselves, each reported distances of 0, p-value- 0 (the p-value is rounded to 0 by MASH and provides statistical confidence in the distance estimates rather than in isolate relatedness), and 10000/10000 MASH shares. Mash shares are also known as matching-hashes or min hashes, the more similar two genomes the more min hashes they share. The lower the distance estimate and the more mash shares, the more related the isolates. VRE 1 (1) ST80 and VRE 2 (1) ST80

chromosomes report a distance estimate of approx. 0.002 with 9140/10000 MASH shares, meaning they are highly related – low distance and high mash shares - but not identical (like the first three rows).

VRE 1 (1) ST80 and VRE 2 (1) ST80 plasmid replicon types report a distance estimate of approx. 0.009 with 6981/10000 Mash shares, meaning they're less related to each other – higher distance and lower number of Mash shares. Thus, these two isolates have high chromosomal genetic similarity but less plasmid genetic similarity.

Escherichia coli BSI isolate relatedness via core genome phylogenetic analysis.

A pangenomic analysis was performed on the *E. coli* isolates as they were the most abundant of the total BSI isolates. In total, there were one thousand nine hundred and thirteen core genes shared between the fifty *E. coli* BSI isolates. These core-genes were used to reconstruct the maximum likelihood phylogenetic tree (figure 3.9). As expected, isolates with the same sequence type, for example *E. coli* ST131 (n=32), cluster together within the phylogenetic tree.

Figure 3.9: Maximum-likelihood phylogenetic tree based on the core genome of fifty *E. coli* Bloodstream infection (BSI) Isolates. *Citrobacter freundii* M92 was used as the outgroup to root the phylogenetic tree. The Inner circle color range represent isolates collected from each hospital: *E. coli* isolates colored purple were collected from hospital 3, pink isolates were collected from hospital 2, and blue isolates were collected from hospital 1. The outer circle color range represent isolate sequence types/ serotypes as shown by the color legend on the left. Bootstrapping was done to provide confidence to each tree branch. The bootstrap values are represented by the dark purple dots upon each branch. The smaller the dot the less supported that branch. iTol was used to display the phylogenetic tree.

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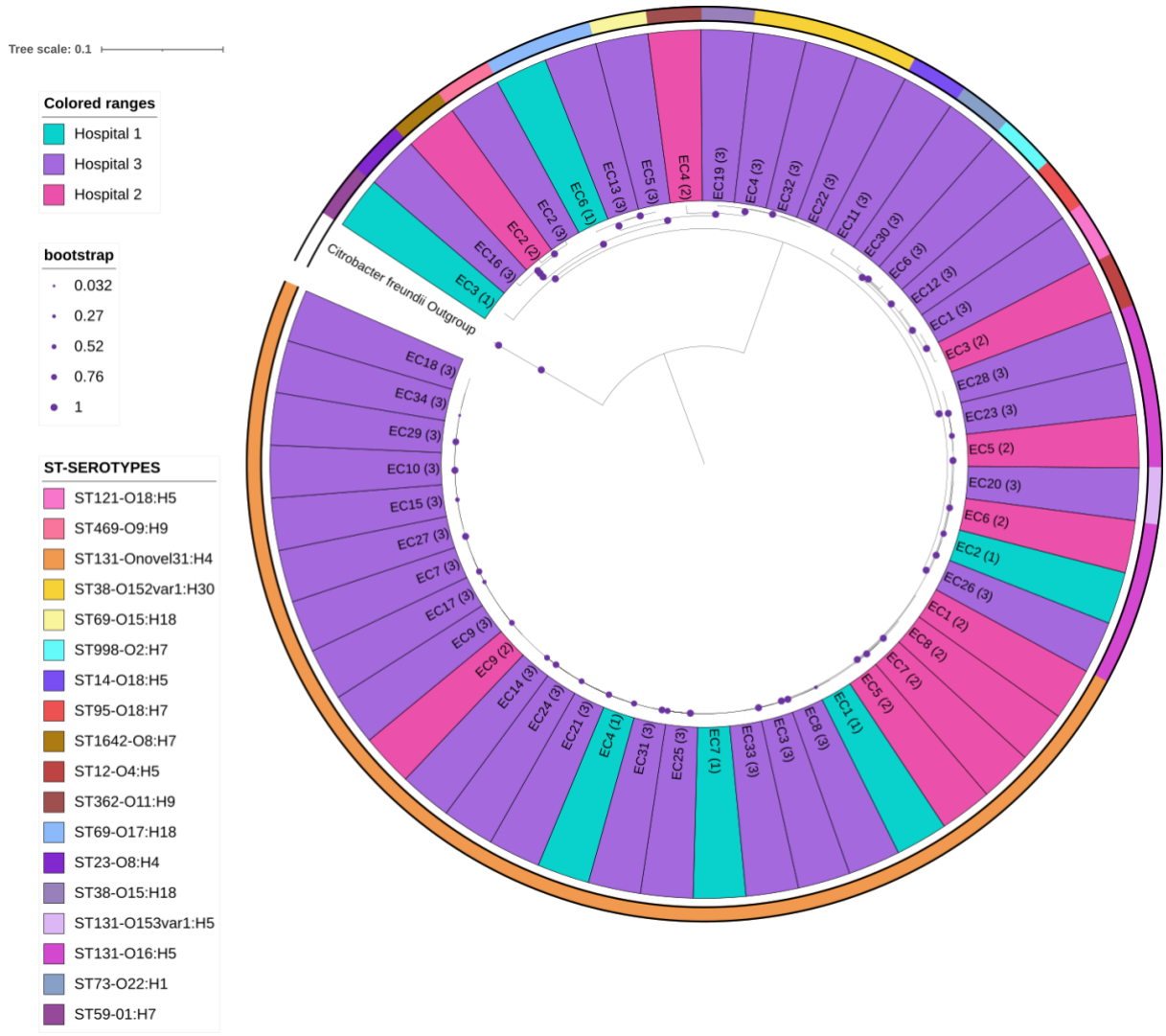
Tree scale: 0.1
Colored ranges
Hospital 1
Hospital 3
Hospital 2
bootstrap
0.032
0.27
0.52
0.76
1
ST-SEROTYPES
ST121-O18:H5
ST469-O9:H9
ST131-Onovel31:H4
ST38-O152var1:H30
ST69-O15:H18
ST998-O2:H7
ST14-O18:H5
ST95-O18:H7
ST1642-O8:H7
ST12-O4:H5
ST362-O11:H9
ST69-O17:H18
ST23-O8:H4
ST38-O15:H18
ST131-O153var1:H5
ST131-O16:H5
ST73-O22:H1
ST59-01:H7
Citrobacter freundii Outgroup
EC3 (1)
EC16 (3)
EC2 (2)
EC2 (3)
EC6 (1)
EC13 (3)
EC5 (3)
EC4 (2)
EC19 (3)
EC4 (3)
EC32 (3)
EC22 (3)
EC11 (3)
EC30 (3)
EC6 (3)
EC12 (3)
EC1 (3)
EC3 (2)
EC28 (3)
EC23 (3)
EC5 (2)
EC20 (3)
EC6 (2)
EC2 (1)
EC26 (3)
EC1 (2)
EC8 (2)
EC7 (2)
EC5 (2)
EC1 (1)
EC8 (3)
EC3 (3)
EC33 (3)
EC7 (1)
EC25 (3)
EC31 (3)
EC4 (1)
EC21 (3)
EC24 (3)
EC14 (3)
EC9 (2)
EC9 (3)
EC17 (3)
EC7 (3)
EC27 (3)
EC15 (3)
EC10 (3)
EC29 (3)
EC34 (3)
EC18 (3)

Discussion

This study shines light on the genotypic and phenotypic landscapes of eighty bloodstream infection (BSI) isolates collected from three Irish hospitals (Hospital 1, Hospital 2, Hospital 3). Comparing and characterizing both Gram (-) and Gram (+) BSI isolates unveiled the predominant spread of *E. coli* ST131:Onovel31:H4 strains, the dissemination of ESBL and CP producing genes among these strains and across hospitals and uncovered isolate relatedness and similarities. The findings of this study provide novel information on ST131 Onovel31:H4 strains including their similarities across the three hospitals, provides support on the ST22 MRSA presence within Irish clinical settings, and uncovers some hospital-acquired infection (HAI) associated ST80 VREfm strains. The data from this study can aide medical professionals understanding the resistance mechanisms from these pathogenic isolates, in addition to providing insight into their probable plasmid mediated transmission of ESBL and CP enzymes.

Escherichia coli invasive infections remain at high levels world-wide with previous studies reporting *E. coli* as the dominant cause of bloodstream infections (Reza and Cormican, 2017; Underwood *et al.*, 2024). We found a similar case within our study; *E. coli* was the most abundant species isolated accounting for sixty percent of our BSI isolates. Our isolates were then sequence typed, with ST131 *E. coli* strains predominating. *Escherichia coli* infections can range from moderate to severe depending on which pathogenic strain has triggered the infection; ST131 *E. coli* strains have been documented as the most prevalent pathogenic lineage throughout Europe (Nicolas-Chanoine, Bertrand and Madec, 2014; Mazzariol, Bazaj and Cornaglia, 2017; Koreň *et al.*, 2023; Kohlenberg *et al.*, 2024), and across the globe (Johnson *et al.*, 2010; Jafari *et al.*, 2020; Li *et al.*, 2021).

All ST131 isolates were assigned the phylogroup B2 and separated by three distinct serotypes: Onovel31:H4, O16:H5, and O153var1:H5. Interesting, the newly emerged, Onovel31:H4, were the most abundant ST131 variant in our study. Globally, ST131 O25:H4 strains have dominated (Peirano and Pitout, 2010, 2019; Kurittu *et al.*, 2022; Zakaria, Edward and Mohamed, 2022) making the emergence of these niche

Onovel31:H4 strains unexpected. While few studies have reported or analysed the ST131:Onovel31:H4 (Adator *et al.*, 2020; Shawa *et al.*, 2021), ST131 O16:H5 have been reported in clinical studies from various countries (Johnson *et al.*, 2010; Dahbi *et al.*, 2014; Zhong *et al.*, 2015; De Toro *et al.*, 2017; Welker *et al.*, 2020; Kurittu *et al.*, 2022; Kohlenberg *et al.*, 2024), including Ireland. At present, this is the first study in Ireland to report ST131:Onovel31:H4 isolates circulating Irish hospitals. It is not known why this isolate has appeared in Irish hospitals though one theory is that the isolate could have been carried in by visitors or patients that have been out of the country. *E.coli* are frequently associated with ESBL production (Birgy *et al.*, 2016; Merino *et al.*, 2018; Göpel *et al.*, 2025). Extended spectrum beta-lactamases inhibit third generation cephalosporins. These enzymes are highly diverse and have evolved into various family types. The cefotaximase (CTX) type family of ESBLs are currently predominant throughout Irish clinical settings. Information regarding CTX-M-type ESBL production within Enterobacteria in Ireland has been described previously by (Morris *et al.*, 2009). Similar patterns of ESBL production co-occurring with high levels of ciprofloxacin and trimethoprim resistance was seen in our isolates. Furthermore, we seen the same moderate levels of gentamycin resistance. Of the ESBL enzymes produced in our isolates, $bla_{CTX\text{-}M\text{-}15}$ was the most frequently reported. This enzyme has dispersed across the globe and has been reported most frequently in Irish environmental and clinical studies over the past five years (Hooban *et al.*, 2022; Farrell *et al.*, 2023; Perestrelo *et al.*, 2023; Prendergast *et al.*, 2023; Kovarova *et al.*, 2025)

In addition to the carriage of ESBL genes, ST131 isolates have been reported to produce high numbers of virulence factors. In comparison and similar to a study reported by Kim et al, there was no significant difference in the number of virulence factors present across all our *E. coli* isolates regardless of sequence type (Kim, Kim and Lee, 2022). A notable virulence factor reported within our study was *senB*, which encodes the shiga-toxin shET2 and has been associated with cases of severe diarrhea. This gene has been reported in studies from Mexico (Magaña-Lizárraga *et al.*, 2019), Korea (Seo, Do and Lee, 2023), Sweden (Matussek *et al.*, 2017), and a recent Irish study evaluating AMR *E. coli* from pig farms (Ekhlas *et al.*, 2023). Another virulence factor of importance was the secreted autotransporter *sat* gene. The *sat* gene is involved in immune evasion (Freire *et*

al., 2022) and a previous study reported its cytotoxic function (Vieira *et al.*, 2020). One concerning biocide resistant gene, *qacEdelta1*, was acquired by over half of the *E. coli* isolates across all three hospitals. Conferring resistance to both disinfectants and antiseptics, previous studies have highlighted its association with ESBL-producing Enterobacterales (Pastrana-Carrasco *et al.*, 2012; Hounmanou *et al.*, 2021; Asare Yeboah *et al.*, 2024). Investigating plasmid replicon types within isolates uncovered incompatibility (Inc) and Colicin (Col) type plasmids. The highest number of replicon types were reported in the ST131:Onovel31:H4 isolates across all hospitals, with the majority found to be Inc-type. The replicon type, IncFIB (AP001918)_1 or IncFIB was the most frequent plasmid replicon type that seemed to circulate not only across hospitals, but across *E. coli* sequence types/serotypes. IncFIB has been described previously to carry ESBL genes, two of which were reported in our isolates, $bla_{CTX\text{-}M\text{-}27}$ and $bla_{CTX\text{-}M\text{-}15}$ (Cherubini *et al.*, 2022). While long-read sequencing would need to be performed to know for sure, there is a high probability that this IncFIB plasmid carries the ESBL $bla_{CTX\text{-}M\text{-}15}$ gene, similar to studies previously described (Wang *et al.*, 2016; Rocha-Gracia *et al.*, 2022). Evaluating replicon types within the *Klebsiella* isolates found that carbapenem resistant isolates carried the IncL/M(p-OXA-48) replicon type. With $bla_{OXA\text{-}48}$ found within these isolates, similar to studies reported by (Power *et al.*, 2014; Getino *et al.*, 2022) it is possible the carbapenemase gene is present on this plasmid replicon type.

MRSA BSI infections are still prevalent today as they were in the 1960's although it is noted that these infections have been decreasing across the globe and throughout Ireland (Kim *et al.*, 2013; Duerden *et al.*, 2015; Deasy *et al.*, 2019; Boal *et al.*, 2024). High rates of ST22 isolates are associated with both the United Kingdom and Ireland (Shore *et al.*, 2014; Baldan *et al.*, 2015; Harrison *et al.*, 2016; Broderick *et al.*, 2021). Fifty percent of our MRSA isolates (n = 12) were typed as ST22. One defining feature of MRSA isolates is the mobile genetic element: staphylococcus cassette chromosome *mec* (SCC*mec*) ('Classification of Staphylococcal Cassette Chromosome *mec* (SCC *mec*): Guidelines for Reporting Novel SCC *mec* Elements', 2009). This chromosomal complex allows for cloning sub-typing and combined with sequence types and/or spa-types, aides in the investigation of MRSA clone dissemination on a global scale. Our ST22 isolates (Table 3) were sub-typed SCC*mec*_type_IV(2B) or ST22-IV. Previous studies have highlighted the

ST22-IV epidemic within Irish hospitals (Shore *et al.*, 2010; Creamer *et al.*, 2012; Broderick *et al.*, 2021). Other reports have noted the increased levels of ST22-IV isolates within hospitals/hospital wastewater across the globe (Niek *et al.*, 2019; Silva *et al.*, 2022; Benvenga *et al.*, 2024). The other 50% of our isolates were typed ST5, ST1, ST97 and ST8. To evaluate the different SCCmec complexes, the SCCmecFinder tool was used. However, this tool, available on the Center for Genomic Epidemiology website, is limited to certain SCCmec types, and has reportedly had problems with ST5 MRSA isolates (Kaya *et al.*, 2018). In our study the ST5 isolates were the only isolates without a SCCmec_type detected, but were spa typed as t311 and t9676. The *mecA* gene was reported in all of MRSA isolates across all three hospitals.

These isolates produce a range of virulence factors- toxins, adhesins, leukocidins, and evasions. In our isolates, the *icaA/B/C/D* adhesin operon was reported in all MRSA. As previously studied, the expression of this operon results in strong biofilm formation which inhibits antimicrobial activity, and limits neutrophils eradicating these isolates (Fitzpatrick, Humphreys and O'Gara, 2005; Zalipour *et al.*, 2016; Mohammad, 2022). One component of the cytotoxic Panton-Valentine leukocidin (PVL) was reported in 46% of the MRSA isolates. The gene: lukF-PV occurred with the *icaA* operon in eleven isolates including ST5 (n =5), ST97 (n =3), ST8 (n =1) and ST1 (n =2). Notably, the second component, lukS-PV was not reported in any of the MRSA isolates. This indicates that PVL is not functional within our isolates, as the two components are needed to activate its cytotoxic activity against receptors on various immune cells (Adler *et al.*, 2006; El Haddad and Moineau, 2013; Otto, 2013). Other toxins reported within our isolates include the *Staphylococcal* enterotoxins (*sec, selk, selq*) which are commonly associated with food poisoning (Ortega *et al.*, 2010; Etter *et al.*, 2020), and gamma-hemolysins (*hlgA, hlgB, hlgC*), which have been reported to target receptors on immune cells, similar to PVL (Rahman, Izaki and Kamio, 1993; Pivard *et al.*, 2023).

In the case of VREfm, infections caused by these isolates increased within Ireland over the last decade. In comparison, our study found a low levels of VREfm in contrast to other BSI isolates and previous studies (Ryan *et al.*, 2015; Whelton *et al.*, 2016; Egan *et al.*, 2022). Though it is noted that our study includes isolates from three Irish hospitals and

not the whole of Ireland. Vancomycin-resistant *E. faecium* isolates were all sequence typed ST80 and belong to the clonal complex CC17. ST80 isolates have been disseminated world-wide, and some studies have highlighted their association with the *vanA* resistant gene rather than *vanB* (Lee *et al.*, 2018; Pratama *et al.*, 2021). In line with these reports, the *vanA* operon was present across all our isolates.

To evaluate genomic relatedness between both the chromosomal and plasmid landscapes of each isolate, MASH distancing was utilized. For MASH the smaller the distance the more related the isolates. Previous taxonomy studies have utilized the MASH distance method (Argimón and Aanensen, 2016; Abram *et al.*, 2021; Aswal, Singhal and Kumar, 2023). Like a study reported by (Lipworth *et al.*, 2022) we used MASH to compare our BSI isolates. The findings of our MASH analysis revealed the presence of highly related ST131-Onovel3:H4 strains collected across all three hospitals. Interestingly, the chromosomal data indicates the bacteria clustered within their sequence/serotypes, with Onovel31:H4 strains more like each other than O16:H5 strains even though they are both ST131 pathogenic lineage. The findings of our plasmid data highlight the spread of plasmid-mediated resistance between isolates regardless of sequence/sero types and locations. Our data suggests the probable movement of the IncFIB plasmid possibly carrying ESBL genes between not only the bacterial species, but their subsequent sequence types. Similar methods were used by (Matlock *et al.*, 2023) in their study, when investigating the plasmid sharing between Enterobacterales collected from human and livestock samples.

Conclusion

The findings of this study report on the dissemination of ST131:Onovel31:H4 *E. coli* BSI isolates throughout Irish hospitals. These strains are novel to Irish clinical studies and have surpassed the frequently reported ST131 O25:H4 pathogens. Plasmid characterisation highlights the IncFIB plasmid replicon type, which was possibly circulating through different *E. coli* sequence/ serotypes and across the three Irish hospitals, with a high probability of harbouring the ESBL enzyme, $bla_{CTX\text{-}M\text{-}15}$. A high prevalence of the MRSA isolates was of the ST22 lineage, these strains are known to circulate around Irish and English hospital environments, and the *mecA* gene was harboured by all the MRSA isolates. Both *E. coli* and MRSA BSI isolates displayed several metal resistant, biocide resistant and virulence genes aiding in their pathogenicity, and protection against disinfectants and antimicrobials.

Chapter 4: Final Discussion

Final Discussion

Antimicrobial resistance (AMR) is a global one-health crisis and one of the biggest threats to human life (Abimbola, Otieno and Cole, 2021; Walsh et al., 2023; Aslam et al., 2024; Cutrupi et al., 2024; Hamilton et al., 2024). The impact of AMR within hospital environments has been previously described (Ferrara et al., 2024; Lim et al., 2024; Ravi and Singh, 2024; Lakhani, Jindal, and Khatri, 2025). Hospitals in themselves are like a double-edged sword. At the same time, they house and treat patients, they are multi-operational environments in which hundreds of people come into contact with each other on an hourly basis, increasing pathogen transmissions to and from the hospitals, there are high levels of antimicrobial usage, and medical devices/ surfaces that act as perfect reservoirs for bacterial biofilm formation (Devanga Ragupathi et al., 2022; Bouhrour, Nibbering and Bendali, 2024; Lordelo et al., 2024). These are just some of the forces driving AMR within hospitals although interventions from Infection Prevention and Control teams (IPC) such as, monitoring antimicrobial use, improved hand hygiene, and locating bacterial reservoirs have been implemented to help combat AMR (Storr et al., 2017; Tomczyk et al., 2021; Brink and Richards, 2022).

The findings of chapter two of this thesis shed light on pathogen reservoirs within Irish hospital sanitary ware and uncover the ESBL and CP producing microbial populations residing within these hospital showers, sinks, and toilets. The results within our study from isolates collected in 2021, were like a recent Irish study conducted by Wu *et al*, in which they investigated ESBL producing bacteria isolated from Irish hospital wastewater pipes. Similarly using 16SrRNA microbial sequencing to identify these pathogens, Wu *et al*, discovered large populations of *S. maltophilia* within sanitary ware. Interestingly and like our study, *S. maltophilia* were the most abundant species isolated from these wastewater pipes, although Hospital-acquired *S. maltophilia* infections for Ireland are less than 1%. Our study found two more species from the same genus, *S. pavanii* and *S. geniculata*. Both species are beneficial in bioregulation. Our study is the first to note their presence in Irish hospital sanitary ware. Although neither are considered as dangerous as *S. maltophilia*, *S. geniculata* has been highlighted as a potential pathogen (Flores-Alvarez *et al.*, 2024).

When comparing methods, Wu *et al*, utilised methods different to our study, these included species identification with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) and evaluating the biofilm formation of their *S. maltophilia* isolates. They further tested *S. maltophilia* isolates producing strongly adherent biofilms against various disinfectant agents, concluding not only differences in biofilms between *S. maltophilia* isolates but that in both cases Optizan™ eradicated these isolates stronger than bleach (Wu et al., 2025). In our study we focused on the AST profiles of our *S. maltophilia* isolates. Interestingly we found some of our isolates displayed resistance against TMP/SMX (n= 22%). TMP/SMX is the optimal treatment against infections caused by this bacterium, with resistance reported world-wide as rare. Antimicrobial breakpoints are difficult to determine for *S. maltophilia*. the investigation into different disinfectants is an option for the eradication of the *S. maltophilia* populations present within hospital sanitary ware. These methods, evaluating biofilm formation, are useful and could provide a deeper understanding of our *S. maltophilia* populations if utilised.

To the best of my knowledge, I have found that our study and the (Wu et al., 2025) study are the only two to have identified and discussed *S. maltophilia* reservoirs within Irish hospital sanitary ware.

The findings of chapter three of this thesis characterised eighty bloodstream infection (BSI) isolates collected from patients across three Irish hospitals. These results bring to light various resistant mechanisms within ESBL and CP producing Enterobacterales, MRSA, and VREfm isolates. Data from this thesis support previous studies in highlighting the ST131 endemic within the Irish clinical and natural environments. This is evident by the high frequency in which these ST131 isolates are being reported and associated with the carriage of the CP enzyme, bla_{OXA-48}, and ESBL enzyme, $bla_{CTX-m-15}$.

(Morris et al., 2012) were the first to detect ST131-O25bH4 harbouring bla_{OXA-48} implicated in an infectious outbreak within an Irish medical ward. During the same year, (Burke *et al*., 2012) reported the large abundance of ST131-O25b isolates cultured from long-time care facilities within Dublin. A study by (Ludden et al., 2020) further investigated the

spread of ESBL producing ST131 isolates within Irish long-term care facilities and found that ST131 clade C clones predominated. One limitation of our study was that we didn't separate our ST131 isolates by clades, although we see similarly that their ST131 isolates frequently harboured *bla*$_{CTX\text{-}M\text{-}15}$. An epidemiological study reported the dissemination of ST131 isolates carrying CP enzymes across EU/EEA countries and reported 101 *bla*$_{OXA\text{-}48}$ positive ST131 strains collected within Ireland from 2016 to 2024. This study further reported both O16:H5 and O25:H4 serotypes as the most prevalent ST131 strains (Kohlenberg et al., 2024).

Results from our study unveiled some O16:H5 isolates but there were no O25:H4 isolates detected. Our findings highlighted the dissemination of ST131-Onovel31:H4 strains within the Irish hospital environment. These ST131 strains have not been reported in previous Irish studies and appear novel to the Irish hospital environment.

While the findings discussed in chapter two are from data collected from the hospital sanitary ware of one Irish hospital compared to the findings of chapter three which revolve around pathogens that have already infected patients and were collected across three Irish hospitals, there are some similarities between the data. Firstly, sanitary ware swabs were collected from Hospital 2 during 2021, and some BSI isolates were collected from Hospital 2 in 2024. One sanitary ware isolate that underwent whole genome sequencing was identified as *E. coli* and sequence typed ST131 (SW ST131). This *E. coli* ST131 isolate harboured the carbapenemase *bla*$_{OXA\text{-}48}$ and an IncL plasmid replicon type which is suspected of carrying this antimicrobial resistant gene. Similarly, several of the BSI isolates from Hospital 2 were sequenced typed ST131 (BSI ST131), although of these isolates the ESBL enzymes were reported rather than carbapenemase enzymes. Investigating the CPE infections for hospital 2 showed zero infections of CPE for 2021. This indicates than even though CP producing *E. coli* ST131 was present within the hospital showers it hadn't infected anybody.

The phenotypical resistant profiles between SW ST131 and BSI ST131 were alike, as all isolates displayed high levels of resistance to cefotaxime, ceftazidime, ciprofloxacin, and trimethoprim. The second similarity between the sanitary ware isolates and BSI

isolates was the presence of the IncL/M(pOXA-48) _1_pOXA-48 or IncL plasmid replicon type. It is noted for the BSI isolates, that IncL was reported in hospital 3 rather than hospital 2 and within *Klebsiella* species isolates (*K. aerogenes* ST404 and *K. pneumoniae* ST39) both of which harboured bla_{OXA-48}. Within our sanitary ware isolates four *Citrobacter freundii* ST116 and the one *E. coli* ST131 strains were positive for both the IncL plasmid, and bla_{OXA-48} gene. Again, this is interesting as there were no CPE infections reported within hospital 2 for 2021.

A limitation within our study was time. The timeframe for this study was shorter than a PhD, allowing for two years rather than four. To investigate the *S. maltophilia* isolates in more detail, future work should utilize methods like (Wu et al., 2025) in addition to using whole genome sequencing like a study performed by (Korotetskiy et al., 2022) to assemble and annotate the *Stenothrophomonas* genome. This allows for the investigation into its intrinsic / adaptive resistant mechanisms.

If possible, more work should extract the IncL carbapenemase plasmid from the *C. freundii* ST116, *E. coli* ST131, *K. aerogenes* ST404, and *K. pneumoniae* ST39 isolates and send each plasmid for long-read sequencing. This part of the study could be performed by following methods described previously (Hidalgo et al., 2019) to reconstruct each plasmid, detect the bla_{OXA-48} gene and other metal/biocide/antimicrobial resistant genes acquired by this plasmid. Furthermore, running a MASH analysis will determine plasmid similarity, and it could be used to investigate the possibility of identical plasmids found across isolates. Future work could follow the same steps in isolating the IncFIB (AP001918) _1 or IncFIB plasmid from the BSI isolates to investigate the probability of it harbouring the $bla_{CTX-M-15}$ gene.

In conclusion, the studies performed in this thesis provide information on AMR pathogen populations present in Irish hospital sanitary ware in addition to highlighting the intrinsic/ adaptive resistant mechanisms of BSI isolates across three Irish hospitals. The largest microbial population discussed were of the species *S. maltophilia*, although infection rates remain low in Ireland. *E. coli ST131* isolates were the most abundant BSI isolates,

with the ARG, *bla*$_{CTX\text{-}15}$, the most common ESBL reported within these isolates. The findings of this thesis may aid the infection, prevention, and control teams within Irish hospitals, in understanding the AMR microbial populations present in the hospitals.

Chapter 5: Bibliography

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Chapter 6: Appendix

Appendix 1

Appendix 1: Antimicrobial Susceptibility testing results for the Enterobacterales isolates mentioned in Chapter 2 (Figure 4) of this thesis. Enterobacter spps are highlighted blue, Escherichia coli is highlighted green. *C. freundii* = *Citrobacter freundii*. Colours: Purple = Resistant, Gray = Intermediate (ATU for EUCAST), Orange = Susceptible. Antimicrobials: IMP = imipenem, ETP = ertapenem, MEM = meropenem, CTX = cefotaxime, CAZ = ceftazidime, CIP = ciprofloxacin, CN = gentamycin, AK = Amikacin, W = Trimethoprim, COL = Colistin.

Identity	Room	Location	Imp	ETP	CTX	CAZ	MEM	CIP	AK	W	CN	COL
C. freundii	C	Shower	21	24	12	19	25	18	18	0	13	2
Enterobacter spp	C	Shower	18	15	0	13	20	18	20	21	12	2
Enterobacter spp	C	Shower	18	16	0	11	20	18	19	20	10	4
C. freundii	C	Shower	18	21	9	0	25	15	17	0	0	1
C. freundii	C	Shower	18	22	10	0	24	15	18	0	0	1
C. freundii	C	Shower	18	21	10	0	24	17	10	0	8	2
C. freundii	C	Shower	18	21	9	0	23	18	10	0	0	8
C. freundii	C	Shower	18	22	8	0	25	18	18	0	0	4
C. freundii	C	Shower	20	21	9	0	23	18	21	0	0	2
C. freundii	C	Shower	19	22	10	8	25	18	18	0	0	1
C. freundii	C	Shower	19	22	10	0	24	17	20	0	8	1
C. freundii	C	Shower	19	22	11	0	24	20	20	0	8	1
C. freundii	C	Shower	19	25	10	0	22	18	18	0	0	2
C. freundii	C	Shower	19	22	10	0	26	18	20	0	0	16

C. freundii	C	Shower	19	22	10	0	24	17	20	0	0	0.5
Enterobacter spp	C	Shower	23	20	15	20	27	17	22	0	12	8
C. freundii	A	Shower	20	23	9	0	25	19	18	0	0	8
C. freundii	A	Shower	18	20	8	0	23	17	18	0	0	2
C. freundii	A	Shower	18	22	10	8	23	18	20	0	0	1
C. freundii	A	Shower	22	16	0	0	21	17	19	23	13	8
Enterobacter spp	A	Shower	24	25	25	23	27	19	21	26	13	4
C. freundii	A	Shower	20	24	8	20	26	21	19	0	13	16
C. freundii	A	Shower	22	25	9	17	27	23	18	0	15	2
C. freundii	A	Shower	21	26	12	18	26	21	19	0	14	2
C. freundii	A	Shower	17	19	13	19	21	21	21	0	14	4
C. freundii	A	Shower	21	25	14	19	27	22	20	0	14	16
C. freundii	A	Shower	26	17	14	19	19	21	20	0	14	2
C. freundii	A	Shower	22	24	8	18	26	21	20	0	13	1
C. freundii	A	Shower	21	25	12	18	27	22	21	0	15	2

C. freundii	A	Shower	18	22	9	16	23	19	17	0	11	2	
C. freundii	A	Shower	17.5	20	0	14	21	18	16	0	13	2	
C. freundii	A	Shower	19	22	0	16	23	21	16	0	13	2	
C. freundii	A	Shower	27	29	11	15	32	20	18	0	12	1	
C. freundii	A	Shower	14	15	16	15	16	19	18	0	12	2	
C. freundii	A	Shower	15	16	11	15	17	20	17	0	13	2	
C. freundii	A	Shower	12	15	9	15	18	20	16	0	11	1	
C. freundii	A	Shower	18	21	0	13	23	19	16	0	11	16	
C. freundii	A	Shower	18	21	0	15	23	19	15	0	13	16	
C. freundii	B	Shower	15	16	13	29	18	20	17	0	12	2	
C. freundii	B	Shower	27	28	10	10	33	14	17	0	0	2	
C. freundii	B	Toilet	19	21.5	9	18	24	15	17	0	10	4	
C. freundii	B	Toilet	18	21	0	17.5	24	17	16	0	10	2	
C. freundii	B	Toilet	20	22	9	17	24	19	17	0	12	2	
C. freundii	A	Shower	15	16	10	15	17	20	18	0	13	1	

C. freundii	A	Shower	17	22	9	15	23	14	16.5	0	10	2
C. freundii	A	Shower	18	22	8	16	23	20	18	0	12	8
C. freundii	A	Toilet	15	16	10	15	17	20	18	0	13	2
C. freundii	A	Toilet	19	22	11	17	24	20	18.5	0	13	2
C. freundii	A	Toilet	19	23	11	16	24	25	18	0	11	4
C. freundii	A	Toilet	15	17	11	17	20	21	18	0	13	2
C. freundii	A	Toilet	18	23	8	17	23	20	17	0	13	2
C. freundii	A	Toilet	20	21	0	16	24	20	18	0	13	1
C. freundii	A	Toilet	27	27	8	16	30	20	16	0	13	2
C. freundii	A	Shower	21	23	10	18	25	20	18	0	15	16
C. freundii	A	Shower	20	22	10	16	24	21	20	0	14	2
E. coli	A	Shower	18	21	0	13	23	19	16	0	12	1
C. freundii	A	Shower	18	22	8	18	24	20	18	0	11	2
C. freundii	A	Shower	23.5	22	9	15	23	19	18	0	12	8
C. freundii	A	Shower	18	20	0	15	22	18	17	0	12	8

C. freundii	A	Shower	20	21	0	17	25	20	18	0	13	2
C. freundii	A	Shower	19	22	0	18	25	20	18	0	13	2
C. freundii	A	Shower	19	22	0	17	24	19	18	0	13	2
C. freundii	A	Shower	19	22	0	17	25	21	19	0	11	4
C. freundii	C	Sink	20	22	0	18	25	19	18	0	12	16
C. freundii	C	Sink	20	22.5	10	20	23	18	18	0	12	4
C. freundii	C	Sink	20	23	9	20	26	19	17	0	13	4
C. freundii	C	Sink	20	23	9	19.5	27	20	16	0	10	2
C. freundii	C	Shower	17	21	7	0	23	25	18	21	17	16
C. freundii	C	Shower	17	19	7	0	23	25	17	22	15	2