Characterisation of antimicrobial resistance in hospital wastewater populations with in-depth analysis of *Klebsiella oxytoca*

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Glossary of abbreviations

AA	Amino acids
AMR	Antimicrobial Resistant
ARG	Antimicrobial Resistant Gene
BNZ	Benzalkonium Chloride
BP	Base Pairs
CAZ	Ceftazidime
CHL	Chlorhexidine
CIP	Ciprofloxacin
COL	Colistin
ECOFF	Epidemiological Cut Off Value
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FEP	Cephalosporin
GEN	Gentamicin
IMI	Imipenem
IU	International Units
KO	*Klebsiella oxytoca*
LEV	Levofloxacin
MALDI-TOF	Matrix-assisted Laser Desorption/Ionization of Time of Flight
MDR	Multidrug Resistant
MIC	Minimum Inhibitory Concentration
MLST	Multi Locus Sequence Type
NCBI	National Centre for Biotechnology Information
PCA	Principal Component Analysis
PS0	*Pseudomonas* Agar

RND	Resistance-nodulation-division
ST	Sequence Type
Terg0	Tergitol-7 without antibiotics
TergC+	Tergitol-7 with ciprofloxacin
TOB	Tobramycin
TRI	Triclosan
WGS	Whole Genome Sequencing
WHO	World Health Organisation
WW	Wastewater
XLD	Xylose Lysine Deoxycholate agar

Declaration

I declare that this thesis presents work carried out by myself and does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; to the best of my knowledge, it does not contain any materials previously published or written by another person except where due reference is made in the text; and all substantive contributions by others to the work presented, including jointly authored publications, is clearly acknowledged.

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Summary

The development and dissemination of antimicrobial resistance is one of the most concerning issues currently facing society. An action plan was endorsed by the World Health Organisation to combat the issue of antimicrobial resistance which emphasised the need to "strengthen knowledge through surveillance". Recent reports by the WHO have named *Enterobacterales* as Critical 1 Priority requiring research but gaps in literature exist regarding the emerging, opportunistic *Enterobacterales* species which are highly capable of harbouring and disseminating antimicrobial resistance. Wastewater surveillance as a tool to track antimicrobial resistance, has huge potential however, most studies focus on genomic analysis of resistance genes without culturing and testing organisms to account for the discrepancy between genotypic and phenotypic resistance often observed. This study used phenotypic and genotypic analysis to compare the three sites receiving wastewater from three different departments within one hospital- Radiology/Oncology, Residential Wards, Theatre/Outpatient/Emergency and a community site as a reference site.

The objective of this study was i) to establish methodologies for monitoring the antimicrobial and biocide resistome within hospital wastewater and determine their potential for use as antimicrobial resistance surveillance and ii) to determine the genotypic resistance determinants of emerging, opportunistic pathogens of concern, *Klebsiella oxytoca*.

A comprehensive review of the literature is presented in **Chapter 1** exploring antimicrobial resistance and the drivers of resistance development and dissemination. Specifically, this review presents the relationship between selective pressures and its influence on bacterial ecological niches. General methods employed within this study are described within **Chapter 2**. In **Chapter 3**, laboratory-based methods were used to isolate and phenotypically analyse resistant bacterial populations within the wastewater sites. This study recovered 1065 organisms from a total of 102 different species. The most prevalent being *Pseudomonas spp*. (n=216), *Klebsiella spp*. (n=170). Multidrug resistance was prevalent amongst all species isolated from wastewater with 84% *C. freundii*, 73% *E. cloacae*, 44.4% of *E. coli*, 59% of *K. pneumoniae* and 67% of *K. oxytoca* isolates multidrug resistant. This study revealed distinct differences in sensitive or resistant populations at the different hospital wastewater sites. This is seen distinctly in *K. oxytoca* where 56% and 82% of isolates from the Reference site and hospital Residential ward were completely antibiotic

sensitive, in contrast to 97% of isolates from Radiology/Oncology and Theatre/Outpatient/Emergency being multidrug resistant. Finally, this study supported the notion that multidrug resistance is associated with resistance against other antimicrobials, finding 72% of multidrug resistant isolates were also resistant against 1 or more biocides. Chapter 4 genotypically characterised *K. oxytoca*, an emerging Enterobacterales of highly ubiquitous within the environment. Two of the eight, sequenced isolates belong to an unknown and unassigned sequence type. Resistance determinants conferring resistance against the 3rd and 4th generation cephalosporins were found, namely, bla_{TEM} and $bla_{SHV\text{-}12}$. Furthermore, this is the first study to find *K. oxytoca* resistant to a common biocide, triclosan, carrying a *fabV* gene. Finally, we report mutations in GyrA, a topoisomerase protein, within four sequenced *K. oxytoca* isolates were phenotypically resistant (MICs >2mg/L) against ciprofloxacin. A Gly409Ala mutation was observed at position 87, and this has not been previously reported or characterised, as such, it is unknown if it plays a role in increased fluoroquinolone resistance. **Chapter 5** presents a general discussion of the overall findings of this study, in the context of current literature and proposes future directions.

Chapter 1. Introduction

1.1. Review of the literature

1.1.1 The golden era of antibiotics

The World Health Organisation (WHO) has identified antimicrobial resistance (AMR) among the top 10 global health threats, posing a significant threat to the health of humans, animals and the environment [1]. A recent report revealed almost 5 million deaths are attributed to AMR worldwide and this is estimated to rise to 10 million annual deaths by 2050 [2, 3]. However, this current and grim threat has only been considered in recent years, arisen from a very different era and perception of antibiotics. The discovery of penicillin in 1928, hailed as the "greatest medical breakthrough"[4], revolutionised medicine and introduced the 'golden age' of antibiotic discovery for the subsequent three decades [5]. Antibiotics are widely available, naturally or synthetically derived compounds that kill or inhibit bacterial growth, and have extended the average human lifespan [6] by treating infectious diseases and enabling a plethora of medical interventions and treatments. Antibiotics are considered "pillars of modern medicine" and it is not possible to imagine modern medicine without them [6, 7].

1.1.2 Emergence of antimicrobial resistance

AMR resistance was noted quickly; after the initial discovery of penicillin, antibiotics became quickly and widely available but within six years of clinical use, multidrug resistant *Staphylococcus aureus* isolates were discovered to be resistant against not only penicillin but also to six other antibiotic classes [8]. Shortly after, resistance was detected in *Pneumococcus spp., Streptococcus spp., Enterococcus spp.* [9, 10]. Since the 'golden era of antibiotics', bacterial resistance rates have severely surpassed the rate of drug discovery [1, 9, 11-13]. Antibiotic resistant bacteria require treatment with alternative antibiotic families, however, multidrug resistance (MDR), where bacteria are resistant to three or more antibiotic classes, severely limit available treatment options [12, 14, 15]. MDR organisms are unfortunately widely prevalent [15-17] and present the modern day with the dangers of a time prior to antibiotic discovery. Furthermore, current globalisation and ease of travel has seen the rapid and ongoing dissemination of dangerous antimicrobial resistant and MDR organisms and determinants worldwide [18-20].

1.1.3 Resistance to non-antibiotic antimicrobials and cross resistance

Antimicrobials are agents, such as antivirals, antifungals and antibiotics that kill or inhibit the growth of microorganisms; other antimicrobials, including biocides, disinfectants, heavy metals, may co-select for antibiotic resistance with many studies finding bacteria with resistance to both antibiotics and biocides [17, 21, 22]. These antimicrobials are used in everyday household products and in healthcare to inhibit or slow down the growth of bacteria [11, 23]. Cross resistance is bacterial resistance to different antibiotic classes or distinct antimicrobial agents through a single molecular mechanism [24]. For instance, expression of the MexCD-OprJ efflux system can confer resistance to a common biocide, triclosan, in *P. aeruginosa*, this expression has been linked to cross resistance in fluoroquinolones, namely ciprofloxacin [17, 25]. Another alarming example of cross resistance is the overexpression of MexAb-OprM in *P. aeruginosa*, which confers resistance to a common preservative benzalkonium chloride and is linked to cross resistance to fluoroquinolones, cephalosporins and aminoglycosides [17]. In *K. pneumoniae*, resistance to a common disinfectant, chlorhexidine selects for resistance against a last line antibiotic, colistin, through the upregulation of smvA [26]. A 2022 study demonstrated the CuS-CusR efflux system, previously thought to give bacteria resistance against copper and silver ions, is also able to confer resistance against tigecycline in *K. pneumoniae* [27]. Thus, when considering bacterial resistance, it is important to consider resistance to not only antibiotics, but also to other types of antimicrobials and the dangers of cross resistance.

1.1.4 The burden of antimicrobial resistance

AMR has an enormous global burden. AMR carries a huge economic burden, currently, the management of AMR costs $300 billion and this cost is projected to reach over $1 trillion by 2050 [3]. Furthermore, AMR impacts healthcare immensely, drug resistant related infections prolong hospitalisations, affecting recovery outcomes and patient quality of life [7]. AMR impacts morbidity and mortality; it is estimated 5 million annual deaths are attributable to bacterial AMR and of that, 1.3 million deaths are attributable to MDR bacteria [2]. Unless urgent action is taken, the increasing resistance rates and ever limited therapy choices will be responsible for 10 million annual deaths a year by 2050 [3].

1.1.5 Factors that influence the rate AMR development

Antimicrobial resistance genes (ARG) exist naturally, well predating the global use of antibiotics; resistant bacteria have been found in the natural environment in undisturbed

places [28-30] and have been discovered in permafrost dating back 30,000 years, [30]. Antibiotics exist naturally and are produced by bacteria for increased competitive survival, predation and signalling and as such, the development of resistance was a fitness advantage, necessary for bacterial protection against antibiotics and thus, bacterial survival [31].

A direct relationship exists between antimicrobial use and the increased emergence of resistance [11]. Antibiotics are heavily integrated into healthcare and the overuse, misuse and abuse of these is thought to be a large contributor to the fast emergence of AMR and MDR. Consumer overreliance on antibiotics has repeatedly been demonstrated amongst patients in all cultures, believing antibiotics can cure viral related infections and as a result, place demand upon prescribers [32]. The inappropriate and overprescribing of antibiotics is prevalent in hospitals and especially in primary care environments [32].

Antibiotic use is not a concern only in healthcare but is widely used in other areas including veterinary practice and agriculture [33]. Globally, the agricultural use of antibiotics is significantly higher than within clinical use, especially in intensive farming where antibiotics are used prophylactically in feed or as growth promoters [34]. The overuse of antibiotics within agriculture has now also been recognised as a major contributor towards AMR development [33].

Anthropologic activity has led to antimicrobial residues in the general environment at concerning concentrations [35, 36] and numerous studies have shown a positive link between antibiotic usage, contamination and its effect on antibiotic resistance [37, 38].

1.1.6 Introduction of stewardship and a global action plan

The indiscriminate use of antibiotics has propelled the development of resistance and as a result of antibiotic misuse, antimicrobial stewardship programmes have been implemented [12, 39, 40]. Antimicrobial stewardship is the careful management of antimicrobials within healthcare through activities, strategies and interventions, intended to improve patient safety through the encouragement of appropriate antimicrobial use [40]. To address this serious global threat of AMR, the WHO endorsed an action plan to help combat the issue of AMR [1, 41]. This plan sets out 5 main strategic objectives: 1) *'Improving the awareness and understanding of AMR'*; 2) '*Strengthen knowledge and awareness through surveillance and research'*; 3) '*Reduce infection through sanitation, hygiene and infection prevention'*; 4) '*Optimise the use of antimicrobial medicines*' and 5) "*Strengthen knowledge through*

surveillance" [41]. In Australia, antimicrobial stewardship was introduced within the last decade as an essential criterium for hospital accreditation [42]. The 2020 Antimicrobial Stewardship Clinical Care Standards stewardship program aims to optimise the use of antimicrobials to decrease the rate of AMR development [40].

This cross-industry reliance on antibiotics has led to the adaptation of a One Health plan, by the Australian Department of Health and Aged Care and the Department of Agriculture and Water Resources [33]. The One Health approach coordinates and collaboratively works with areas across human health, animal health, food, pharmaceuticals, and agricultural industries to reduce the rate of AMR development [33].

Unfortunately, despite the implementation of antimicrobial stewardship and of collaborative One Health plan, the use of biocide remains unregulated an its inappropriate use needs to be addressed in order to help decrease AMR.

1.2. Antibiotic mechanisms of action

Antibiotics are classified into different classes based on their molecular structure and mode of action. The five most commonly prescribed antibiotic classes in Australia are beta-lactams, quinolones, macrolides, tetracyclines and aminoglycosides [**Table 1**] [43]. Antimicrobial drugs act on a variety of targets and are either bacteriostatic, inhibiting further growth of the bacteria or bactericidal, with the ability to kill bacteria. Their major mechanisms of actions include:

- Inhibition of bacterial cell wall synthesis
- Inhibition or interference of bacterial cell membrane
- Inhibition of interference of DNA synthesis
- Inhibition or interference of protein synthesis
- Inhibition of interference of metabolic pathways

Table 1. Antibiotic classes and their mode of action

Antibiotic class	Examples	Effect	General mode of action
Beta lactams	Penicillin Cephalosporins	Bactericidal	Inhibition of cell wall synthesis

	Carbapenems Monobactams		
Sulfonamides	Sulfamethoxazole	Bacteriostatic	Inhibition of folate metabolism
Aminoglycosides	Streptomycin Neomycin Gentamicin	Bactericidal	Inhibition of protein synthesis
Tetracycline	Tetracycline, Doxycycline	Bacteriostatic	Inhibition of protein synthesis
Chloramphenicol	Chloramphenicol	Bacteriostatic	Inhibition of protein synthesis
Glycopeptide	Vancomycin	Bactericidal	Inhibition of cell wall synthesis
Ansamycin	Rifamycin	Bactericidal	Inhibition of RNA synthesis
Quinolones	Ciprofloxacin	Bactericidal	Inhibition of DNA synthesis
Oxazolidinones	Linezolid	Bacteriostatic	Inhibition of protein synthesis
Lipopeptides	Daptomycin	Bactericidal	Disruption of the Gram-positive cell membrane
Polymyxins	Colistin	Bactericidal	Disruption of the Gram-negative inner membrane

1.3. Bacterial mechanisms of resistance

AMR can be intrinsic, acquired or adaptive [23]. Bacteria can be naturally or intrinsically resistant to antimicrobials, meaning the organism was never susceptible to the agent. Contrastingly, bacteria are said to have acquired resistance when they acquire resistance against an antimicrobial agent they were previously susceptible to.

Resistance can occur through many mechanisms including: i) the inactivation of the antimicrobials through innate enzyme production, ii) the use of active exporters (e.g. efflux

pumps) to expel the antimicrobial, iii) inability for the antimicrobial to penetrate the membrane, iv) the lack of affinity of the antimicrobial to the target site or v) the production of alternative enzymes other than the antimicrobial target, allowing for completion of vital metabolic pathways [Figure 1] [44]. Often, various mechanisms are simultaneously employed in AMR bacteria [23].

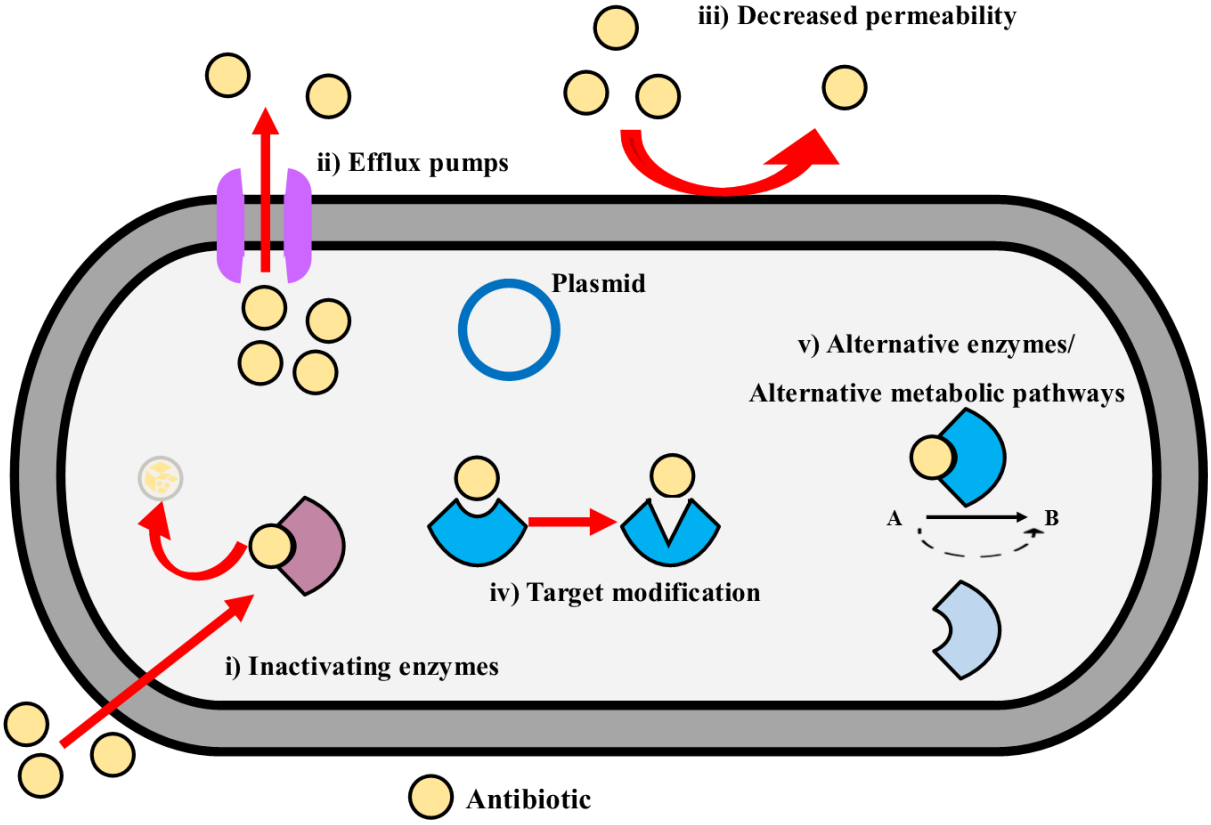

Figure 1. Bacterial mechanisms of resistance

Mechanisms represented here are: i) Inactivating enzymes which inactivate the drug, e.g. β-lactamases ii) Efflux pumps actively transport the drug out of the cell iii) Decreased permeability for example porin mutations can cause a large decrease in the amount of the drug able to enter into the cell and reach the target iv) Target modification whereby the cell modifies the target of the antibiotic such that it loses its affinity to the target v) Alternative enzyme production allows bacteria to continue an essential metabolic pathway that the antibiotic would have otherwise inhibited.

i) Inactivating enzymes

The most prolific example of inactivating enzymes are β-lactamases. [23, 45, 46]. β-lactamases are enzymes which hydrolyse the β-lactam ring, rendering the antibiotic inactive.

β-lactamases are classified into Ambler/molecular classes A, B, C and D based on the similarity of the DNA sequence or functional properties. Classes A, C and D are serine β-

lactamases enzymes whereas class B are metallo-β-lactamases [47]. Generally, Class A is serine-based penicillinases, Class B consists of zinc based β-lactamases, Class C is serine-based cephalosporinases and class D are serine-based oxacillinases [Table 2].

Table 2. Ambler classification of common β lactamases

Class	Type	Characteristics	Example genes
A	Narrow spectrum β lactamases	Hydrolysis of penicillins	*TEM, SHV*
	Extended spectrum β lactamases	Hydrolysis of narrow and extended β lactam antibiotics	*SHV, CTX*
	Serine carbapenemases	Hydrolysis of carbapenems	*KPC, IM*
B	Metallo-β lactamases	Hydrolysis of carbapenems	*NDM, VIM, IMP*
C	Cephalosporinases	Hydrolysis of cephamycins	*AmpC, CMY*
D	OXA-type enzymes	Hydrolysis of oxacillins and carbapenems	*OXA*

KPC: *Klebsiella pneumoniae* Carbapenemase, SME: *Serratia marcescens Enzyme*, IMI, Imipenem-hydrolysing β-lactamase, IMP: Imipenemase type metallo β lactamase, NDM: New Delhi metallo- β-lactamase, OXA: Oxacillinase type metallo β-lactamase.

ii) Drug efflux pumps

Gram-negatives also contains efflux pump complexes which act as active transporters of compounds across the cell membrane. Many pumps are a necessary result of evolution and have led to bacteria's ability to survive and colonise during infections. These pumps decrease the concentration of antimicrobials inside the cell to a sub-toxic level [48] and thus, rendering bacteria resistant against one or multiple classes of antimicrobials. Efflux pumps act as an incredible system of bacterial survival, just one single efflux pump can extrude a variety of structurally dissimilar antimicrobials [49]. There are seven classes of efflux pumps: the Resistance Nodulation Division (RND), the Major Facilitator Superfamily (MFS), the Multidrug and Toxic Compound Extrusion (MATE), the Small Multidrug Resistance pumps (SMR), the ATP Binding Cassette (ABC), proteobacterial antimicrobial compound efflux (PACE) and the drug metabolite transporter (DMT) [50]. RND-type inner membrane proteins form large and complex, tripartite systems with other proteins spanning across the inner, cytoplasmic membrane, the periplasm and the outer membrane [Figure 2].

RND efflux pumps have broad specificity allowing for the development of resistance against multiple antibiotic classes, despite difference in chemical structure [51] and as such, are the most clinically relevant efflux pump in Gram-negative bacteria [48]. One archetypical RND system attributed to MDR is the AcrAB-TolC pump found in *E. coli* and other members of the Enterobacteriaceae family. This efflux pump is has a wide substrate range including antibiotics such as novobiocin, linezolid, macrolides, fluoroquinolones as well as crystal violet, sodium dodecyl sulfate, progesterone and essential oils [52]. The AcrAB-TolC pump is a tripartite pump consisting of three major parts, an Outer Membrane Protein (OMP), a Membrane Fusion Protein (MFP) and an Inner Membrane Protein (IMP) [53]. The structure has been well described by methods such as X-ray crystallography and cellular electron cryo-tomography [54]. The expression of the AcrAB efflux pump is regulated by *acrR*, *marA*, *rob* and *soxS* [54].

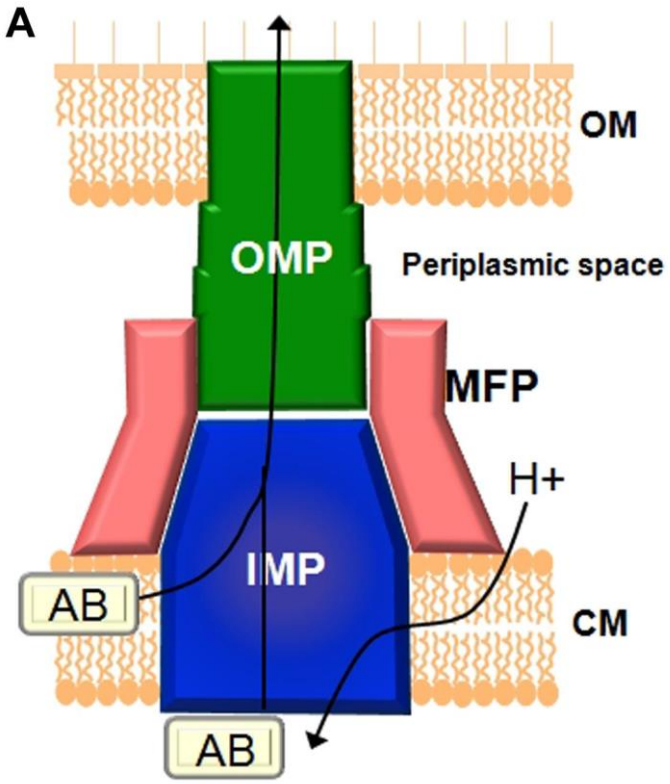

Figure 2. Tripartite drug efflux pump

Schematic representation of a tripartite drug efflux pump showing the inner membrane protein (IMP), outer membrane protein (OMP), in this case, ToIC and periplasmic membrane fusion protein (MFP) [48].

iii) Decreased drug permeability Gram-negative bacteria when compared to Gram-positive bacteria, show higher antimicrobial tolerance due to the presence of an additional outer

membrane acting as a permeability barrier. The permeability barrier of the outer membrane is further reinforced by outer membrane porins (OMPs), protein channels passively allow for permeability of larger hydrophilic molecules across the membrane [55]. Decreased drug permeability in Gram-negative *Enterobacterales* can be through changes to the OMPs through the down regulation of porin expression, replacement of the porin with a smaller channel sized porin and through impairment of porin function [23]. For instance, the replacement of OmpK35 by a smaller porin, OmpK36 results in beta-lactam resistance in *K. pneumoniae* [23].

Decreased drug permeability can also be through the alterations of the outer membrane, for instance, colistin resistance involves decreasing the negative charge present on bacterial surface, through modifications in lipid A, preventing the electrostatic interaction between colistin and the bacterial lipopolysaccharide [23, 56]. This modification is attributed to the overexpression of PmrAB and PhoPQ due to the presence of plasmid-borne *mcr* genes [56].

iv) Target modification

Bacterial resistance can occur through target modification whereby the cell modifies the target of the antibiotic such that it loses its affinity to the target. An example of target modification is seen in fluoroquinolone resistance in Gram-negative bacteria. Fluoroquinolones, such as ciprofloxacin, inhibit DNA topoisomerase II, which is needed for supercoiling, causing DNA fragmentation and cell death [23, 57]. DNA gyrase and topoisomerase IV enzymes, comprised of *gyrA/gyrB* and *parC/parB* genes, are also essential for supercoiling; thus, resistance in Gram-negative organisms is often associated with mutations in *gyrA/gyrB* or *parC/parB* which alter protein structure and lead to bacterial resistance through decreasing the binding affinity of fluoroquinolones [23, 57, 58].

ii) Alternative enzymatic/metabolic pathways

Bacterial resistance can also occur through alternative enzyme production allowing bacteria to continue an essential metabolic pathway that an antibiotic would have otherwise inhibited. For instance, β-lactam resistance can occur via the alteration of the target penicillin binding protein (PBP) where Methicillin resistant *Staphylococcus aureus* (MRSA) is a well-known example of this. The *mecA* gene on the staphylococcal chromosomal cassette (SCCmec), encodes for a modified PBP - referred to as PBP2a which

is less sensitive to β-lactam binding and has the same, necessary enzymatic activity as PBP and thus, continues to aid the normal synthesis of the cell wall, increasing its survival ability [23, 45, 46].

1.3.2 Vertical and horizontal gene transfer

These mechanisms can be a result of adaptive mutations of target sites, such as mutations in genes including *gyrA*, *gyrB*, *parE* and *parC* to combat quinolone resistance or mutations changing functional systems, such as increased or decreased expression of influx or efflux or decreased permeability [52]. Most often, multiple mutations are present in isolates with a high level of AMR [59]. What makes AMR development so rapid and concerning is bacteria's ability to acquire resistance through two major mechanisms, by spontaneous chromosomal mutations (known as vertical gene transfer) or by horizontal gene transfer; through either transformation, transduction or conjugation [Figure 3]. Genes conferring resistance are carried on integrons, plasmids and transposons; horizontal gene transfer allows for the very rapid spread of resistant genes amongst different bacterial species. It also allows for the rapid emergence and spread amongst not only pathogenic bacteria but also amongst commensal and environmental bacteria [44]. It is likely these traits have all contributed to the global emergence of AMR and MDR.

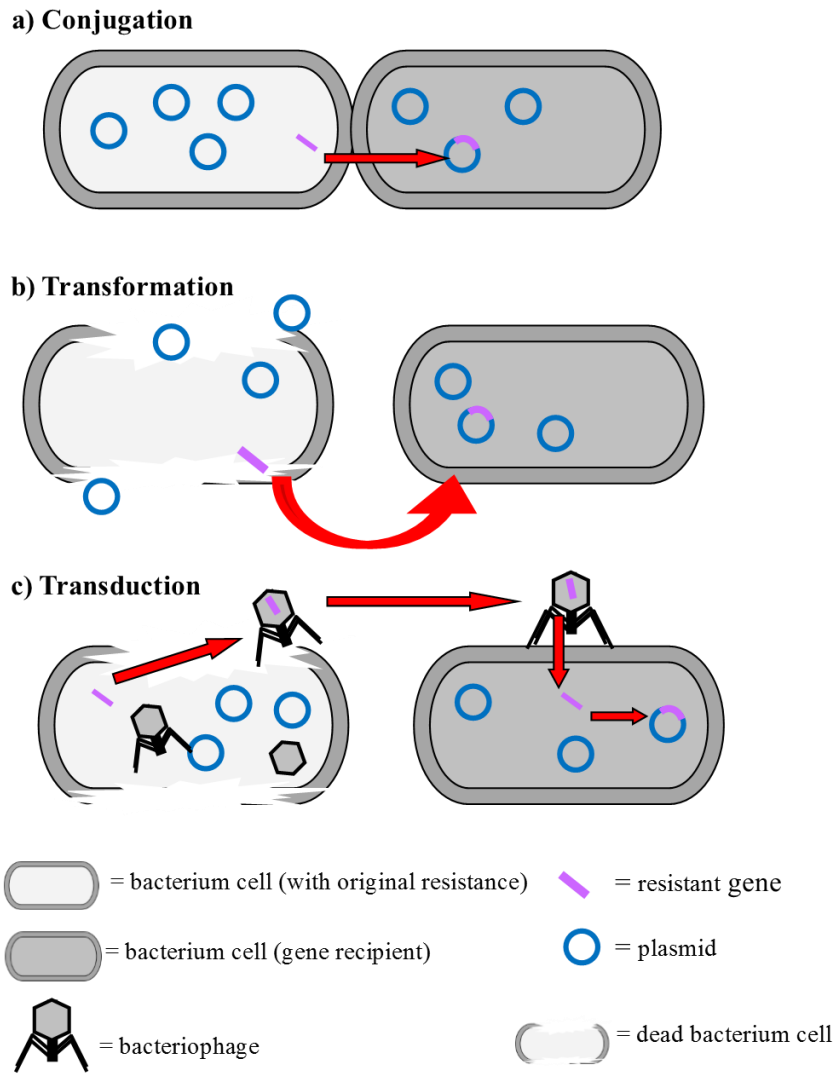

Figure 3. Mechanisms of bacterial gene transfer

a) Conjugation showing bacterial genetic transfer through direct contact, b) Transformation showing horizontal gene transfer of new genetic material disseminated from one bacterium, into the environment and into a new bacterium and c) Transduction showing viral transfer of genetic material.

1.4 Mobile genetic elements play a major role in acquired resistance and ARG dissemination

The bacterial pan-genome consists of core genomes, shared amongst all individuals within a clade, and accessory genome, a set of genes unique to a particular strain [60]. The accessory genome component is able to acquire genetic elements from non-parental sources through bacterial mobile genetic components such as integrons, insertion sequences and plasmids[18, 61]. Through this horizontal gene transfer, bacteria are thus able to rapidly obtain or disseminate genes conducive to their survival, including AMR genes.

1.4.1 The role of plasmids in acquired resistance and ARG dissemination

Plasmids are circular replicons thought to be responsible for majority of acquired resistance to antibiotics; mobility can be between plasmids themselves or from a chromosome to a plasmid. Dangerously, many AMR conferring plasmids are able to control their replication and can be conjugative, able to transfer not only themselves but other plasmids or they can be mobilizable, where the plasmid is small and able to be transferred by conjugative plasmids [62]. They may also harbor genes which can encode for MDR efflux pumps. Plasmids carrying AMR genes are often conjugative. The common classification of plasmids is based on the genetic relatedness of four incompatibility groups (Inc): IncF, IncP, Ti and IncI. IncF includes IncF, IncS, IncC, incD and IncJ; IncP includes IncP, IncU, IncM and IncW; Ti includes IncX, IncH, IncT and IncI includes IncI, IncB and IncK [63]. The most common groups shown to confer AMR genes include IncA/C, IncH, IncF and IncI [64]. Due to their association with extended beta lactamase (ESBL) genes, IncF (45-200kb in size) plasmids are the most described in literature and are found in *Enterobacterales*, most commonly in *E. coli*. Common genes found on IncF include bla_{CTX}, bla_{TEM}, bla_{NDM}. The IncH is largest in size (75-400kb) and can be found in a large array of hosts including the *Enterobacterales, Aeromonas, Vibrio* and *Yersinia* families [65].

1.5. Biocides and heavy metal resistance constitute a big threat

An overlooked and underestimated contributor to the development of AMR is the widespread use of biocides and heavy metals [66, 67]. Biocides are broad spectrum antimicrobials able to act against bacteria, fungi and viruses and include antiseptics and disinfectants such as triclosan, chlorhexidine and benzalkonium chloride. Triclosan, is a widely used antimicrobial, estimated 4.5 million kilograms of triclosan is used annually worldwide and is found in personal and healthcare products such as within hand sanitisers and toothpaste [68]. Another common antiseptic, chlorhexidine, is widely used in clinical settings for example, as pre- and post-operative decontamination, in healthcare products such as dental mouthwashes [68]. Benzalkonium chloride is an antimicrobial preservative, widely used from healthcare, such as in intranasal medications, in household products, including in personal health and beauty products, laundry products and within antimicrobial soaps and in agricultural tools such as within water storage tanks [69]. Biocides are so ubiquitous in human everyday lives and heavily relied upon as antimicrobials, as such, reduced bacterial efficacy and the rise of resistance is a matter of high concern.

Bacterial resistance to biocides has been described as early as the 1950's; however, in more recent decades, links between biocide resistance and antibiotic resistance have been increasingly observed [70, 71]. There is a growing concern biocides and heavy metals may co-select for antibiotic resistance with many studies finding bacteria with resistance to both antibiotics and biocides [21]. Experiments have also shown when bacteria are adapted to be resistant against biocides in the laboratory, there is an increase in antibiotic resistance [26, 72] andeven very low levels of biocides could select for antibiotic resistance [73]. For instance, the upregulation of efflux pumps correlates with benzalkonium chloride resistant *P. aeruginosa* from different ecological niches and found to also give rise to cross resistance across multiple families of antibiotics [17]. Decreased susceptibility to biocides, role of efflux pumps and correlation with antibiotic cross resistance has been demonstrated in other organisms such as *A. baumannii* [74, 75]. Biocide and heavy metal resistance is a problem across all industries including healthcare, food production and agriculture such as fisheries and farms [76].

Until more recent years, antimicrobial stewardship had not been considered but the rising issues of cross resistance, especially in clinical settings has brought this issue to light. Genes conferring resistance to one antimicrobial, may confer resistance to others; studying mechanisms of resistance is not a straightforward process as numerous resistance determinants can act in synergy to increase the level of resistance to a particular compound.

As previously mentioned, selective pressures are strongly correlated with cross and co resistance with antibiotics. *K. pneumoniae* was the first reported *Enterobacterales* with colistin resistance in 2004 [77] and has since been reported with co-resistance against colistin in 29% of carbapenem resistant strains compared with 3% in carbapenem sensitive *K. pneumoniae* [78].

1.5.1 Factors affecting triclosan resistance

Triclosan (2,4,4-trichloro-2-hydroxidephenileteretere is a chlorinated phenol widely used as a disinfectant and in beauty and healthcare products. Studies looking at complex aquatic environments have shownexposure to subinhibitory levels of triclosan may act as an additional stressor to the microbial community and may favour cross resistance to other compounds [79, 80]. The effects of triclosan on antibiotic resistance profiles in a complex environment and the concentration of triclosan that selects for resistance is still unknown [81]. McMurry, et al, 1998 first described the inhibition of a enoyl-acyl carrier protein

reductase (*fabI*) in *E. coli* by triclosan and this has since been described across many other species, such as in *S. aureus*, where overexpression of FabI reduces susceptibility to triclosan [82, 83]. The *fabI* gene codes for a protein required in the last step for the elongation cycle in type II fatty acid biosynthesis. Resistance against triclosan has been shown to commonly be due to target modification of FabI, membrane alterations and efflux pumps. Production of FabI isoenzymes: FabL, FabK or FabV can function like the FabI protein and are not affected by triclosan. Thus, an organism such as *P. aeruginosa* with homologues of *fabV* has been observed to be more than 2000-fold more resistant against triclosan [84]. Alterations of *fabI* have not been shown to have any correlation with multidrug resistance, Cameron, et al., 2019, for instance, looked at *E. coli* and *Enterococcus* species in wastewater and did not find any correlation between triclosan resistance and resistance against any antibiotics or heavy metals [85]. This is despite many *fabV* being encoded close to homologs of *zupT* (a gene involved in heavy metal transportation), or other efflux pumps associated with heavy metal tolerance. FabV homologs may have originated from *Aeromonas spp*., and *Yersinia spp.* and further work into these or similar organisms may reveal more about triclosan resistance [86-88]. Although the presence of altered *fabI* homologues has not been associated with multidrug resistance, the upregulation of nonspecific efflux pumps when exposed to triclosan has been observed to cause increased tolerance and cross resistance to certain classes of antibiotics [89, 90].

1.5.2 Factors affecting benzalkonium chloride resistance

Quaternary ammonium compounds (QACs) are organic cationic compounds used very widely in hospital and hygiene or cleaning related products. They act by binding to the negatively charged bacterial cell wall, disrupting its integrity and causing the release of low molecular weight constituents, ultimately leading to cell lysis and death [22, 91]. Common QACs include benzalkonium chloride and are most usually present in products at a concentration of 0.01-1%. The length of the alkyl chain largely determines its efficiency as an antimicrobial. Studies have shown the presence of sublethal QACs in an environment can increase membrane permeability, increase Reactive Oxygen Species production and thus able to promote horizontal gene transfer of plasmids amongst bacteria. Resistance genes against QACs are commonly found, especially in MDR bacteria; for instance 73% of MDR *A. baumannii* from within an intensive care unit was found to carry the *qacE* gene [92]. As the QAC genes, such as *qacE*, are carried on class 1 integrons, it is one of the likely reasons for co-selection to be present amongst QAC resistant isolates. That is, antibiotic resistance can also be found on Class 1 integrons [93]. QAC resistance genes are

categorised as part of the MFS, eg. *qacA/B*, or the SMR family, eg. *qacC/D*, *qacE*. Furthermore, the overexpression of efflux pumps has been shown to contribute to benzalkonium chloride resistance, namely overexpression of MecAB-OprM and is associated with cross resistance to fluoroquinolones, cephalosporins and aminoglycosides [17].

1.5.3 Factors affecting chlorhexidine resistance

An extremely common disinfectant used widely in healthcare is chlorhexidine (1,6-bis-4'-chlorophenylbiguanide hexane), a divalent, cationic biguanide antiseptic. Chlorhexidine acts by the formation of bridges between anionic sites on bacterial cells and the cationic biguanide groups displace divalent Mg^{2+} and Ca^{2+} cations causing the cell membrane to become leaky. The membrane loses its structural integrity and cell death incurs [94, 95]. Literature has found 12.05% and 7.8% of *K. pneumoniae* and *P. aeruginosa* show resistance against chlorhexidine acetate and has demonstrated a lower level of resistance against common hospital biocides in clinically pathogenic organisms compared with antibiotics. The rate of biocide resistance compared to reports from previous years has increased [92]. Bacteria with increased resistance to chlorhexidine can also have co-resistance to antibiotics across different classes including aminoglycosides, carbapenems, ciprofloxacin and tetracycline [96] and this co-resistance has been demonstrated in other studies [72].

In a study from Wand, et al., 2017, chlorhexidine adapted *K. pneumoniae* carried SNP's in *phoPQ* and/or *smvR* and all *K. pneumoniae* strains with a mutated *smvR* gene demonstrated an upregulation of the *smvA* gene; *SmvR* acts as a repressor of *smvA*, an efflux pump involved in the efflux of QACs. This study suggests chlorhexidine resistance in *K. pneumoniae* may be due to the *smvA* efflux pump [26]. Homologues of the *Tet* repression genes *smvR* and *smvA* may also be found in many other multidrug resistant, Gram-negative pathogens and thus, there may be a similar mechanism of resistance against chlorhexidine in other Gram-negative pathogens [26, 97]. The study by Wand demonstrated the cross resistance between chlorhexidine and colistin [26].

1.5.4 Factors affecting silver resistance

It is not just chemical antimicrobials that should be considered, heavy metals such as silver and copper are often used in healthcare and other settings. A third of all *Enterobacterales* carry *sil* genes; amongst clinical *Enterobacterales*, *E. cloacae* and *K. oxytoca* were the most frequent carriers of *Sil* genes with 58% and 49% of isolates respectively carrying at least one *Sil* gene [98]. *Enterobacterales* can carry all three of the most common *sil* homologs; *silE, silP* and *silS*; this was the case in 29% of *E. cloacae, 23% of K. pneumoniae and 35% of K. oxytoca.* Patients within burn units are at higher risk of exposure to silver resistant *Enterobacterales* [98].

1.6 Selective pressures and the hospital environment

Anthropologic reliance on antibiotics in healthcare environments, where patients have a higher likelihood of carrying pathogenic bacteria, has resulted in widespread antibiotics and its metabolites in the environment [17]. The high load of antimicrobial and antibiotic use in combination, in turn is able to exert a selective pressure on bacterial populations and is a high contributor to the development of AMR and MDR organisms. Healthcare facilities are a hotspot for AMR and MDR development, acquisition, and dissemination, and this includes not only the internal environments, but also the surrounding environment, including healthcare facility wastewater [99, 100].

How an aquatic environment may influence the dissemination of ARG is not yet certain but it is clear hospital wastewater has a higher potential for antibiotic resistance [99, 100] and a higher ecotoxicity [100-102] when compared to urban wastewater. Hospital effluent can contain bacterial populations with a higher resistance profile compared to antibiotic resistant bacteria in urban wastewater [103]. This difference may be due to the antimicrobial selective pressure within the wastewater environment. Antibiotics are not fully metabolised in the human body and their excretion along with the disposal of unused drugs and biocides allows them to be a 'pseudo-persistent' contaminant due to their continuous supply into the environment [104, 105]. Antibiotics at several hundred-times below their inhibitory concentration can select for resistant bacteria [106] and even at concentrations as low as parts per billion in wastewater, may be enough to maintain the antibiotic resistant population and be a suitable environment for the transfer of genes [107, 108]. Further evidence of selective pressure was presented when studies found hospital effluent to contain a higher frequency of antibiotic resistant bacteria compared to community water [100] and resistant organisms in wastewater are found to mostly be MDR [109]. In higher selective pressures, bacterial populations may develop not only antibiotic resistance, but stronger cross resistance against different antibiotic classes [110].

Regardless of how vigorous the treatment process, many pharmaceutical compounds still persist in wastewater treatment plants [111, 112] and as previously mentioned, antimicrobials released into the environment at low concentrations are possibly contributing to the development and rise of resistant populations [106]. A high rate of resistance dissemination from hospital effluent into the general environment has been reported by a number of studies [113-116] and many studies suggest wastewater treatment plants additionally contributes to the spread of ARGs within the environment [117-120]. For example, MDR *Pseudomonas aeruginosa* discharged from a local hospital wastewater treatment plant was found in a Brazilian river used by local residents for cooking and cleaning [114]. Environments and systems receiving water post wastewater treatment, contained an 'alarming' number of ARGs, acting as an ARG reservoir for environmental bacteria [121]. An Australian study found MDR *S. aureus* had survived wastewater treatment and concluded hospital wastewater were hotspots for MDR dissemination into the environment [122].

1.7 Organisms of concern and emerging pathogens

The WHO released the Global Priority Pathogen List, listing 12 bacterial families which pose significant threat to public health. This list acts as a tool to promote research and development into AMR and antibiotic discovery and divides bacteria into Critical, High or Medium priority, based on their propensity to acquire or develop multidrug resistance and cause serious infections. Priority 1, critical category includes carbapenem resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacteriacea [1]*. This is in line with recent reports by the Antimicrobial Use and Resistance in Australia Surveillance System, which noted that there has been significant (7.8% and 63.3%) use of third and fourth generation carbapenem antibiotics, such as meropenem and imipenem, and reports carbapenemase-producing *Enterobacterales* to be the most frequently reported critical antimicrobial resistant family in Australia [123]. Furthermore, the reports emphasises the growing concerns of ESBL producing multidrug resistant organisms, South Australia reported 8.6% and 5.7% of clinically isolated *E. coli* and of *K pneumoniae* are ESBLs respectively [123]. Recent reports by the WHO have demonstrated there is an alarming increase in blood stream infections by ESBL producing *K. pneumoniae*, resistant to 3rd generation cephalosporins or even more concerning, an increase in infections by carbapenem resistant isolates [1].

1.7.1 Klebsiella oxytoca – an emerging pathogen

The *Enterobacterales* family are classified by the WHO as Critical Priority 1 organisms requiring research and development; this family include the *Escherichia spp., Enterobacter spp*., and the *Klebsiella spp*.. *Klebsiella spp*. has become one of the major pathogenic concerns in healthcare due to the increasing emergence of plasmid mediated ESBLs and carbapenem resistance amongst this species [124, 125]. In addition, infections by pathogenic *Klebsiella spp.* with resistance to colistin, a last resort antibiotic, have also been reported and unfortunately these infections have limited remaining treatment options [77, 98]. The *Klebsiella spp*. is a large species consisting predominantly of: *K. pneumoniae,* the *K. oxytoca* complex and *K. variicola*. *K. pneumoniae* contributes up to 95% of all nosocomial infections by *Klebsiella spp*. and this is reflected in literature where *K. pneumoniae* remains the predominant focus [126, 127]. *K. oxytoca* was recently considered to be a complex consisting of nine species, *K. grimontii, K. huaxiensis, K. michiganensis, K. pasteurii, K. spallanzanii, K. oxytoca* and three currently unnamed, novel species of *Klebsiella [126, 128]*. The complex is still referred to within the literature as *K. oxytoca*, and these papers consider pathogenic *K. oxytoca* to be clonally diverse [129]. Current clinical methods are unable to distinguish species within this complex and distinction requires genomic analysis [126, 130]. Even with genomic analysis, many species within the complex have very similar genomic characteristics and either are currently unable to be distinguished from each other or have not been assigned species names yet [130]. Regardless, *K. oxytoca* and the species within the complex are becoming a problem within healthcare [131, 132] and infections by the *K. oxytoca* complex with drug or multidrug resistance are becoming more common [127]. Despite the emerging dangers of the *K. oxytoca* complex, research into *K. oxtyoca* and is still limited and research is heavily focused on the resistance patterns of *K. pneumoniae. K. oxytoca* is ubiquitous in the environment, wastewater, soil, animal gut, handwashing sinks and drains and as such, is classified as an emerging pathogen [133-135]. Its ubiquity in the environment includes hospitals where vulnerable populations reside, and being opportunistic, *K. oxytoca* is responsible for nosocomial infections [133-135]. *Klebsiella spp*. has repeatedly appeared as environmental reservoirs for dissemination of resistant determinants including the beta-lactamase, $bla_{\text{IMP-8}}$ and $bla_{\text{CTX-M}}$ [124] with most common resistance genes in ESBL producing *Klebsiella* a combination of bla_{SHV}, bla_{TEM} and $bla_{\text{CTX-M}}$ [124, 136]. Furthermore, transmission of *Klebsiella* plasmids has been demonstrated to be happening on a global scale with a 2020 study demonstrating the worrying transmission of plasmids carrying carbapenem resistance,

migrating between patients in the Netherlands and in Caribbean [137]. Literature has largely focused on the ability for *K. pneumonia* to disseminate resistance determinants but do not focus on the other prevalent *Klebsiella* species [137]. Previous outbreaks of *K. oxytoca* have been from KPC-producing strains [138] and recent analysis of *K. oxytoca* from an Australian hospital setting found an alarming level of resistance against 3rd generation cephalosporins [127]. Furthermore, there are reported cases of *K. oxytoca* with reduced sensitivity to biocides, including chlorhexidine [77].

Outside of clinical outbreaks, literature contains limited information regarding *K. oxytoca and* the *K. oxytoca complex*. One such gap is in the area of heavy metal and biocide resistance [77]. It remains unknown whether these same resistance systems exist in other *Klebsiella* species and whether they give rise to the same resistance levels.

1.8 A need for AMR surveillance

An action plan was endorsed by the WHO to help combat the issue of AMR and included the need to "*strengthen knowledge through surveillance*" [41]. Tracking the global dissemination of newly discovered ARGs illustrates how rapidly and widely an ARG can spread. An example of this is the novel *β-lactamase* encoding gene, *bla*$_{\text{NDM-1}}$, isolated from an Indian patient in Sweden [139]. Although its discovery was relatively recent, it has since been detected across the world from China, the Middle East and Greece [118, 140, 141]. It has also been found in Vietnam and India in both sewage and tap water and was detected at high levels in hospital effluent [142]. A study by Haller [143] has very recently found that of the four carbapenemase conferring genes tested in hospital effluent, *bla*$_{\text{NDM-1}}$ was one of the most frequently detected. Previously, *bla*$_{\text{NDM-1}}$ had only been found to be predominantly carried by *K. pneumoniae* and *E. coli*, but its rapid dissemination into other pathogenic species is a very significant public health concern [142, 144]. The *bla*$_{\text{NDM-1}}$ gene is just one example of many recently found, novel genes, illustrating the alarming rapidity of global ARG detection and spread.

1.8.1 Surveillance of wastewater for resistant determinants

The COVID-19 pandemic has seen the increased interest in wastewater testing as a surveillance tool for public health [145]. Surveillance through wastewater is an evolving area [146] and the increased interest in wastewater testing for public health transcends into surveillance of AMR markers, to search for genes which may indicate increased bacterial resistance within areas [146-149]. One such method typically used to detect antibiotic resistant bacteria and AMR genes in wastewater relies on whole genome sequencing (WGS) of individual organisms isolated from wastewater. WGS allows for in depth analysis of phylogeny, species evolution, AMR genes, and epidemiology. Microbial genomes can be sequenced with long or short reads produced by sequencing tools such as Illumina's NovaSeq and MiSeq, Ion Torrent, BGI's MGISEQ [150]. Long read sequences, such as those produced by Oxford Nanopore Technologies, are ideal for genomic analysis of plasmids as they produce reads over 30 times longer than short reads, (reads typically >10 kb long), however the cost per sequence, low throughput and inability to be easily analysed means they are not necessarily the most suitable method [151]. Short reads, such as those produced by Illumina, yield reads of 100-300 bp, and is a more readily available method due to their cost effectiveness, high throughput, accuracy and the large number of tools freely available for analysing bacterial short reads [152, 153]. Irrespective of read length, different sequencing technologies have their own advantages and disadvantages in producing accurate, high-quality reads but most importantly, the end user needs to understand the nuances of each technology in order to analyse their data with a level of confidence in the sequence.

Once sequences have been obtained, being able to interpret and analyse the data is another challenge. Visualisation, annotation and alignment tools such as Artemis and Mauve are desktop-based software [154, 155]. There are many commonly available online tools from organisations such as the Centre for Genomic Epidemiology (CGE), Comprehensive Antibiotic Resistance Database (CARD) and the National Centre for Biotechnology Information (NCBI) as well as from many small laboratories [150, 156]. Common phylogeny tools available online include CGE's: NDtree and TreeViewer, iTOL [157]. Openly available online tools for Typing include CGE's: MLST – for interpreting the multi-locus sequence type (MLST) and PlasmidFinder – for finding plasmids in a sequence. Common phenotyping tools available online for bacterial sequence analysis include online tools provided by CGE: ResFinder – for identifying acquired resistance genes against

antibiotics, PathogenFinder- for identifying potential genes which contribute to pathogenicity, VirulenceFinder- for identifying acquired virulence genes [158-160]. Basic Local Alignment Search Tool (BLAST) functions are used to query a particular sequence to search in databases for known and curated genes [161]. BLAST tools give the option of searching through different sequences for instance, many BLAST tools will have at least BLASTP- for searching protein sequences against a protein database, BLASTN- for searching nucleotide sequences against a nucleotide database, BLASTX for searching translated nucleotides against a protein database and BLASTN for searching protein against a translated nucleotide database [162]. NCBI contains the most extensive database but results may match to arbitrary, unpublished data whereas BLAST functions from sources such as CARD use a curated database, often providing a more straight forward result [150].

WGS is unable to explore the complexities of resistance mechanisms in uncultured organisms, for instance, this method would be unable to detect genes that were harboured but not expressed [85]. Finally, genomic methods for surveillance does not account for the phenotypic resistance profile of organisms and gives little information regarding the efficacy of those identified genes. Previous studies found *mcr-9* gene, attributing it's potential colistin resistance in *E. coli* and *Salmonella spp.* but recently studies found *mcr-9* gene played no role in colistin resistance in either organism [163, 164].

1.9 Summary of the project

The development and dissemination of bacterial AMR is one of the most concerning issues currently facing society [22]. Surveillance is an important aspect to combating AMR. Wastewater surveillance as a tool to track AMR, has developed significantly in the past few years. However, most studies tend to focus on genomic analysis of resistance genes without culturing and testing organisms to account for the discrepancy between genotypic and phenotypic resistance often observed. Surveillance has not been used to compare selective pressure between multiple sites of a single hospital. Additionally, AMR has not been well characterised in emerging pathogens, which have shown propensity to be highly multidrug resistant and capable of carrying a plethora of resistance determinants. Finally, while most attention is focussed on antibiotic resistance, biocide resistance and its potential effects have gained less traction in recent decades. Given that reduced susceptibility to healthcare biocides is commonly associated with increased antibiotic resistance, this issue also needs

urgent attention. This study will explore the use of phenotypic and genotypic methods to obtain surveillance data regarding AMR from a hospital wastewater environment.

1.9.1. Aims of the research project

The development and dissemination of AMR is one of the most concerning issues currently facing society [123, 125]. The increased rate of resistance development observed recently is attributed to the overuse of antibiotics and unfortunately, bacterial resistance rates have severely surpassed the rate of drug discovery [5, 8-10]. AMR, and MDR is limiting the availability of drugs able to combat infections. AMR extends beyond resistance to antibiotics but also includes resistance against biocides and heavy metals now ubiquitously found in healthcare and household products [22]. Recent studies show the significance of disinfectants and its contribution towards AMR [26] however, there are currently no global breakpoints or epidemiological cut-off values (ECOFFS) for biocides [91]. Furthermore, AMR has not been well characterised in emerging pathogens, which have shown propensity to be highly multidrug resistant and capable of carrying and disseminating a plethora of resistance determinants.

To address the global threat of AMR, an action plan was endorsed by the WHO to help combat the issue of AMR, including the need to "strengthen knowledge through surveillance" [41]. Wastewater surveillance as a public health tool shows promising for the detection of AMR organisms and their genes. Analysis of the antimicrobial resistome of a healthcare facility captures the situation for the entire facility and could provide insights that would be missed by looking at samples from some patients in the facility. Culture-based techniques for analysis of complex environments are not frequently used, and thus, optimisation of methodology is required [145]. The need to better understand the complex environment of wastewater requires the establishment of surveillance methodologies, especially the incorporation of traditional phenotypic laboratory methods combined with genotypic surveillance.

To address these gaps, the aims for this study are:
Aim 1: To establish methodologies for monitoring the antimicrobial and biocide resistome in hospital wastewater and to determine their potential for AMR surveillance.
Aim 2: To determine the genotypic resistance determinants of emerging opportunistic pathogen of concern, *Klebsiella oxytoca*.

Chapter 2. General materials and methods

2.1 Materials and media preparation

2.1.1 Preparation of selective and differential media

A range of selective and differential media were used to isolate organisms to limit the variety of species obtained. Columbia agar, *Pseudomonas* agar, Bile Aesculin, Tergitol-7 were prepared according to manufacturer's instructions [Table 3]. Unless otherwise stated, all media was steam sterilised in an autoclave at 121ºC for 15 minutes.

Table 3. Selective, differential media used and bacterial species to be identified.

Media	Manufacturer	Supplemented antimicrobial	Concentration (µg/ml)	Target organism
Columbia agar	Oxoid	None	-	All non-fastidious and viable
Pseudomonas agar w. Pseudomonas CN supplement	Oxoid	None	-	*Pseudomonas spp.*
		Meropenem	2	Carbapenem resistant *Pseudomonas spp.*
XLD	Oxoid	Ciprofloxacin	1	Fluoroquinolone resistant coliforms
Bile Aesculin	Oxoid	Vancomycin	4	Vancomycin resistant *Enterococcus spp.*
Tergitol-7 with supplement	Oxoid	None	-	Coliforms
		Ciprofloxacin	1	Fluoroquinolone resistant coliforms

2.1.2 Broth preparation

Dilutions of wastewater was performed in 0.1% Bacteriological Peptone (Oxoid). Minimum Inhibitory Concentrations were performed with Cation Adjusted Mueller Hinton Broth (Difco). After isolation and identification, bacterial isolates were frozen in 15% glycerol broth: Glycerol (Chemsupply) and Nutrient Broth #2 (Oxoid). Luria Broth (Acumedia) was used for the efflux inhibition assay.

2.1.3 Antimicrobial preparation

A range of antibiotics and other antimicrobials were used for the minimum inhibitory concentration experiments: cefepime, ceftazidime, ciprofloxacin, gentamicin, levofloxacin, benzalkonium chloride, chlorhexidine digluconate and silver nitrate were obtained from Sigma-Aldrich- (St Louis, USA). While colistin, meropenem, imipenem and triclosan were obtained from Glentham Life Sciences (Corsham, UK).

2.2 Sample isolation and storage

2.2.1 Sample collection and storage

Wastewater sampling took place on Monday the 20th of July 2017. SA Water personnel provided access and assisted with the collection of samples from three hospital sewer sites: Radiology/Oncology (Site 1), Residential Wings (Site 2), Theatre/Outpatients/Emergency (Site 3) and a reference site away from the hospital (Site 4). Samples of 1 litre were collected into sterile containers every hour from 6 am until 5 pm. Composite samples were made in sterile containers as follows: 5pm- 6am, 6-12pm and 12-5pm and diluted to 10^{-1} in sterile peptone water before they were sent on ice for immediate processing at the University of South Australia. After processing, the remaining samples were then immediately frozen at -20 and kept for later use.

2.2.2 Sample isolation process

Defrosted samples provided to the laboratory already diluted to 10^{-1} , were serially diluted in peptone water (0.1%), performed in duplicate from 10^{-1} to 10^{-4} in a sterile 12 well plate. Each dilution was plated and incubated at 37 °C for 24 hours. After 24 hours, colonies were counted, and colony morphology and characteristics noted. Plates were further incubated for another 24 hours and counting was repeated. Organisms of interest, based on characteristics on the selective media, were carefully picked, streaked onto fresh plates and incubated at 37 °C for 24 hours.

2.2.3 Isolate identification using MALDI-TOF

Representative isolates from each morphology were sub-cultured onto Columbia agar plates, grown at 37ºC for 24 hours and identified the next day by matrix-assisted desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) (Bruker Daltonik,

GmbH, Bremen, Germany). Isolate identification was accepted with score values greater than 1.7.

2.3 Phenotypic analysis of isolates

2.3.1 Antibiotic susceptibility testing with broth microdilution

The minimum inhibitory concentrations (MICs) against antimicrobials of interest were determined using the broth microdilution method, following methodology by EUCAST recommendations [165]. The following classes of antibiotics were used: Aminoglycosides: gentamicin (GEN), tobramycin (TOB); Carbapenems; imipenem (IMI), meropenem (MER), Cephalosporins: ceftazidime (CAZ), cefepime (FEP); Fluoroquinolones; ciprofloxacin (CIP), levofloxacin (LEV) and Polymyxins; colistin (COL). *E. coli* ATCC 25922 was used as a quality control strain. Organisms of interest were grown at 37 °C for 24 hours on appropriately supplemented media, to ensure isolates retained any resistance they carried. From these plates, a log phase was made in Cationic Mueller Hinton Broth (CMHB) for each organism to be tested and grown for 4 hours. A volume of 100µL of CMHB was added to every well of a 96-well microtitration plate, an extra 100µL was added to column 1 and 12. Each antimicrobial was added at double the desired concentration into column 1 and serially diluted through to column 10, where 100µL was discarded. The OD of the log phase was measured at 660 nm and the log broth was diluted with CMHB until the OD was at 0.25, before being diluted a further 100x. A volume of 100µL of the 100x diluted organism was then added to the 96 well plate in all wells besides column 12, which was used a growth control. The plate was read at 595nm for a time zero reading before being incubated on a shaking platform at 37°C for 18 hours. The plate was then read, and results noted. For compounds giving a high time zero reading, viability was assessed using 10µL of 0.1 % resazurin added to all wells and incubated for 15 minutes on a shaking platform at 37 ºC. Resazurin is reduced to a pink colour by metabolically active cells and was used to discern whether compounds were toxic within high time zero wells and ensure the results were a true measure of cell viability. The MIC value was determined as the lowest concentration in mg/L of the tested antimicrobial visibly inhibited growth of the tested organism. Isolates showing resistance to three or more antibiotic classes were considered as MDR [166].

2.3.2 Determination of biocide/heavy metal epidemiologic cut-off values

The MICs of Biocides; chlorhexidine digluconate (CHL), triclosan (TRI), benzalkonium chloride (BNZ) and heavy metals; silver nitrate ($AgNO_3$) were determined using the broth microdilution technique described in 2.3.1. The ECOFF values (with 95% cut-off) of biocides, not yet determined by EUCAST, were established using the ECOFF finder XL 2010 program (https://clsi.org/meetings/microbiology/ecoffinder/).

2.4 Whole Genome Sequencing and assembly

2.4.1 DNA extraction and 16s profiling

Genomic DNA for 16s sequencing was extracted using the DNeasy PowerWater Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. Samples were sent to the Australian Genome Research Facility (AGRF, Melbourne, Australia) for 16s sequencing.

2.4.2 DNA extraction for whole genome sequencing

Genomic DNA was extracted using the NucleoSpin®Microbial DNA kit (Machery-Nagel, Düren, Germany) according to the manufacturer's instructions. DNA was quantified using a NanoDrop™ Spectrophotometer (ThermoFishcer Scientific).

2.4.3 Whole genome sequencing

Genomic DNA was sent for whole genome sequencing (WGS) at SAPathology, sequencing libraries were prepared using Illumina's Nextera XT DNA library preparation kit (Illumina Inc., USA). Briefly, fragmented DNA was fragment was amplified by Nextera XT indices (Illumina Inc., USA) using a low-cycle PCR reaction. Quality control was achieved with positive (Neisseria meningitidis ATCC13077 and negative controls (lysis buffer). The library was then purified and normalised. The Illumina NextSeq 550 platform was used for sequencing with the NextSeq 500/550 Mid-Output kit v2.5 (300 cycles) (Illumina Inc.)

2.4.4 Sequence assembly

Initial assembly and analysis of the *K. oxytoca* sequences was performed by Tormes v.1.1 [167] - an open-source pipeline for WGS analysis directly from raw Illumina paired-end sequencing data. Along with assembly, Tormes also performs other functions such as cleaning and trimming of reads, species identification, gene annotation, pangenome analysis and a search for resistance properties; Trimmomatic [168] removes adapters and filters low

quality reads from the raw sequences. The sequences are then run through SPades [169] which utilises multiple algorithmic variations of the De Bruijn graph method of assembly of the paired end reads. Upon completion of the assembly, Quast [170] is used for analysis of the quality of the assembly. The resulting contigs are analysed using Multi Locus Sequence Typing (MLST) and Kraken [171] to determine the species of the assembled genomes.

2.5 Sequence analysis

2.5.1 Pangenome analysis

Multi-Locus Sequence Type (MLST) assignment was carried out using the MLST software [172] and the PubMLST database [173]. Protein annotation was carried out with Prokka [172]. Tormes [167] has the capability of also including pangenome and phylogenetic analysis and the process in the pipeline is performed by ROARY [174] and FastTree [175]. The final report which Tormes produces is created in R and uses the following packages: ggree, knitr, plotly, RColorBrewer, reshape2, rmarkdown, treeio [176-181] .

2.5.2 Resistance genes

Isolates were screened for AMR genes using ResFinder [182], Antibiotic Resistance Gene-ANNOTation (ARG-ANNOT) [183] and the Comprehensive Antibiotic Resistance Database (CARD) [156]. Resistance determinants against biocide and heavy metals was found using the BacMet database [184]. A manual curation of AMR genes was carried out using Notepad++ to search through annotated Tormes and Prokka files.

2.5.3 Plasmids

The Plasmidbot pipeline, created by Brad Hart (unpublished) was used in the identification of plasmids [Figure 4]. Assembled files were then blasted against NCBI's Blastn PLSDB database, MASH PLSDB database and abricate search against the Plasmidfinder database. The corresponding plasmid .fasta files were then downloaded to create bam files by aligning with the raw WGS fastq files and only alignments matching greater than 80% were then used to create 'consensus' .fasta files. These files were annotated with sequences from NCBI's Plasmids GenBank. The Phylogenetic tree was created by using the NCBI Datasets

utilities to download all available *Klebsiella oxytoca* genomes in NCBI RefSeq database. Using the software tool 'FastANI' all 8 samples were compared against the 154 downloaded *K. oxytoca* refseq sequences.

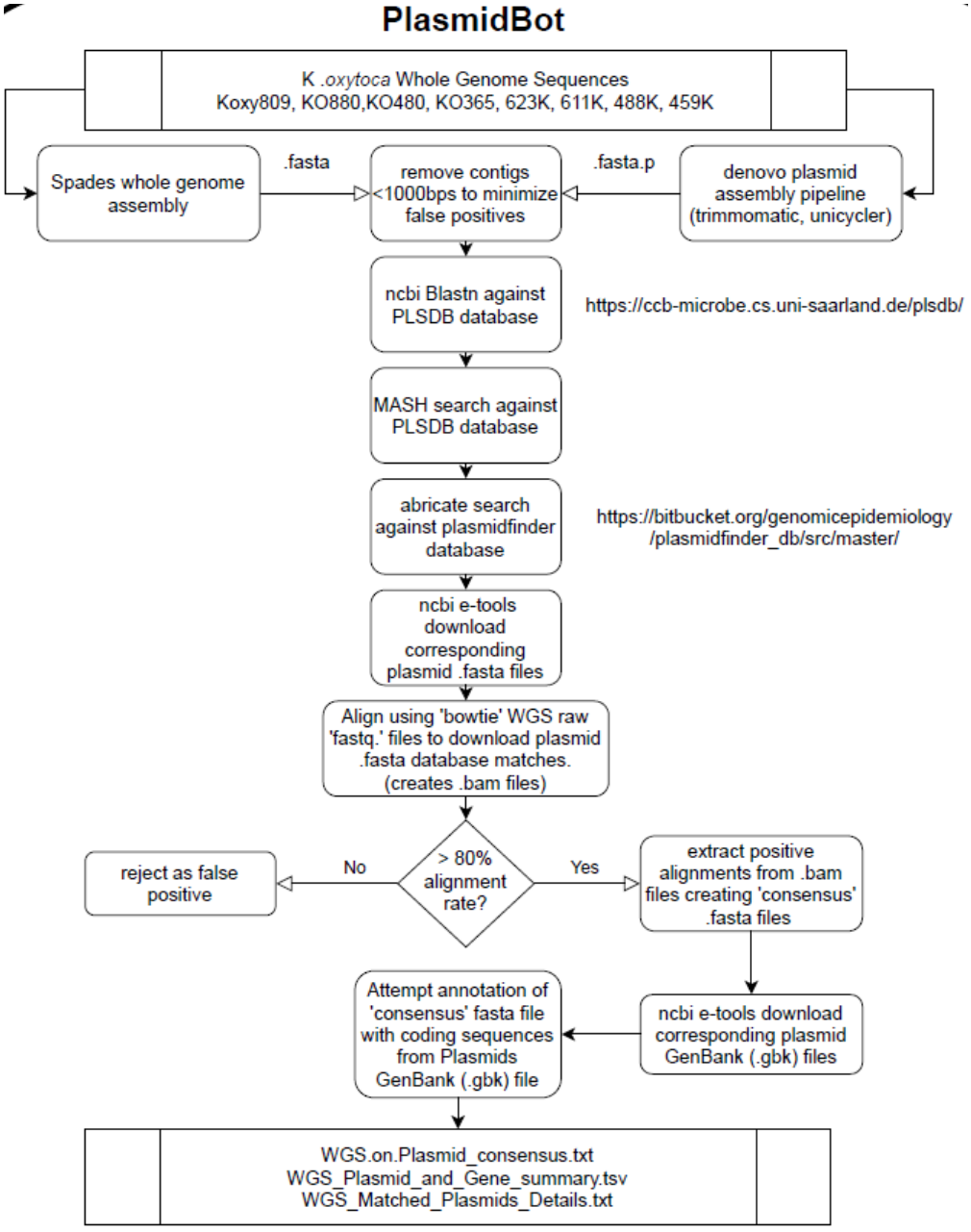

Figure 4. Plasmidbot pipeline

Plasmidbot pipeline methodology as created by Brad Hart (Unpublished).

2.6 Data and Statistical analysis

Data related to the antimicrobial susceptibility profile was entered into spreadsheets (Microsoft Excel 2016; Microsoft Corporation, Redmond, Washington, USA). Statistical analysis was performed using R studio, version 1.3.1073 [185].

Chapter 3. Phenotypic analysis of the resistome of hospital wastewater

Work from this chapter formed part of the following publication: "Amsalu, A.; Sapula, S.A.; De Barros Lopes, M.; Hart, B.J.; **Nguyen, A.H**.; Drigo, B.; Turnidge, J.; Leong, L.E.; Venter, H. Efflux Pump-Driven Antibiotic and Biocide Cross-Resistance in *Pseudomonas aeruginosa* Isolated from Different Ecological Niches: A Case Study in the Development of Multidrug Resistance in Environmental Hotspots. *Microorganisms* **2020**, *8*, 1647.

Subsequent studies have also been based on data and isolates collected as part of this study.

3.0 Introduction

The development and dissemination of antimicrobial resistance (AMR) is one of the most concerning issues currently facing society [123, 125]. AMR, and multi-drug resistance is limiting the availability of drugs able to combat infections and additionally, has a major economic burden [186]. AMR extends beyond resistance to antibiotics but also includes resistance against biocides and heavy metals now ubiquitously found in healthcare and household products [22]. Recent studies show the significance of disinfectants and its contribution towards AMR [26] however, there are currently no global breakpoints or epidemiological cut-off values (ECOFFS) for biocides [91]. Furthermore, AMR has not been well characterised in emerging pathogens, which have shown propensity to be highly multidrug resistant and capable of carrying and disseminating a plethora of resistance determinants.

To address the global threat of AMR, an action plan was endorsed by the WHO to help combat the issue of AMR, including the need to "*strengthen knowledge through surveillance*" [41]. Recent reports by the WHO have named Enterobacterales as Critical 1 Priority requiring research and demonstrated resistance against third generation cephalosporins and resistance against carbapenems within this family are increasing at a concerning rate [1]. Furthermore, in Australia, ciprofloxacin resistance and vancomycin resistant *enterococci* are reported to be now as concerning as MRSA in healthcare [42].

To address the urgent need for AMR surveillance, this chapter used a wastewater-based epidemiological approach to study the resistome of three different sites from one hospital. To create a snapshot of the microbial diversity and resistome within a complex, hospital wastewater environment, microorganisms were isolated, purified, and identified. Subsequently, the phenotypic antimicrobial susceptibility profiles of organisms were

created to gain an understanding of the resistance profiles represented by organisms of interest and to identify multidrug resistant isolates.

To select for the resistant organisms, agar was selected based on their ability to select for these species and supplemented with antibiotics. The use of culture-based techniques for analysis of complex samples for AMR surveillance is not commonplace due to time and labour restraints, and thus, optimisation of methodology was required [145]. The concentrations of antibiotics added to media was based on available ECOFF values for *Pseudomonas spp.* which generally are higher than ECOFF values for *E. coli*. Resistance profiles were established using broth dilution methods for Critical 1 Priority organisms, *C. freundii, E. coli, E. cloacae, K. pneumoniae* and as well as an emerging, opportunistic Enterobacterales, *K. oxytoca*. Finally, diversity between the different wastewater environments was further explored with 16s profiling to identify the major populations present in the different ecological niches.

3.1 Diversity of hospital wastewater isolates

A total of 1065 isolates, consisting of 102 distinct species were isolated from the wastewater samples. As the aim was to isolate Gram-negative pathogens in the WHO's most critical priority (*Acinetobacter spp*. *Pseudomonads*, *Enterobacterales*), representative numbers of colonies were picked based on expected morphologies for those pathogens on selective and differential media. For example, according to the manufacturer, red colonies with bluish zones on Tergitol-7 agar were expected to be *Salmonella, Shigella*, *Proteus* or *Pseudomonas spp*. [Table 4.], but some isolates such as isolates of *Enterobacter*, *E. coli* and *Klebsiella spp.*, sometimes appeared red instead of yellow [Table 4.]. The results illustrated how varied environmental isolate shape and morphologies were from the presumptive, colours indicated by the clinical specimens. Furthermore, many species had varied morphologies; *Klebsiella spp.* isolates appeared as red, orange, yellow or clear organisms [Table 5] compared to the expected yellow colonies. Size, colour and shape were not adequate predictors for presumptive identification. This illustrated that only relying on the manufacturer's expected morphology for presumptive identification was not enough to assume species when exploring complex samples such as wastewater. A representative number of colonies from each of morphology variants were picked for further identification [Figure 9].

3.1.1 Tergitol-7 selects for a large variety of Gram-negative organisms

Tergitol-7, containing 2,3,5-Triphenyl Tetrazolium Chloride (TTC), is a selective and differential media designed for the detection of *E. coli* from water samples and can also be used for detecting faecal contamination in foods. Tergitol-7 medium allows for the differentiation between coliforms (red colonies), *E. coli* (yellow colonies) and *Enterobacter spp*. (green/yellow colonies). TTC inhibits *Proteus* swarming and inhibits the growth of Gram-positive organisms. Due to the presence of bromothymol blue, lactose fermenters, such as *E. coli* and *Enterobacter spp.*, appear yellow. Non lactose fermenters reduce triphenyl-2H-tetrazoliumchloride to insoluble triphenol formazan resulting in red colonies with blue zones.

The use of Tergitol-7 plates to isolate wastewater samples yielded a large variety of organisms across the 4 hospital sites. The wastewater samples were initially plated as dilutions to assist in the counting of numbers and easy recoverability of isolates [Figure 5]. Selective plating revealed each wastewater site had distinctly different morphologies and recoverable colonies, as illustrated in Figure 5 and to be discussed later in this thesis. A total of 549 isolates were recovered representing isolates with phenotypically varied plate morphologies, varying from those with clear edges, target-like colonies, round, irregular and varied level of mucoid appearance. Isolates from the same species also had varied morphologies. For instance, most *E. coli* were yellow with red centres on Tergitol-7 plates, but other morphologies were observed including round, dark red to irregular, orange colonies. As expected, no Gram-positive isolates were found [Table 4].

Table 4. Expected and observed morphologies on Tergitol-7 agar without selective antibiotics.

Organism	Expected morphology	Observed morphology
E. coli	Yellow with red centre	Round, dark red Irregular and orange Round, yellow
Enterobacter spp.	Green/yellow colonies	Round, dark red Dark purple with clear edge Orange
S. typhimurium	Red with blue zone	None found
P. mirabilis	Red with blue zone	None found
P. aeruginosa	Red with blue zone	Round, red centred with clear edge,

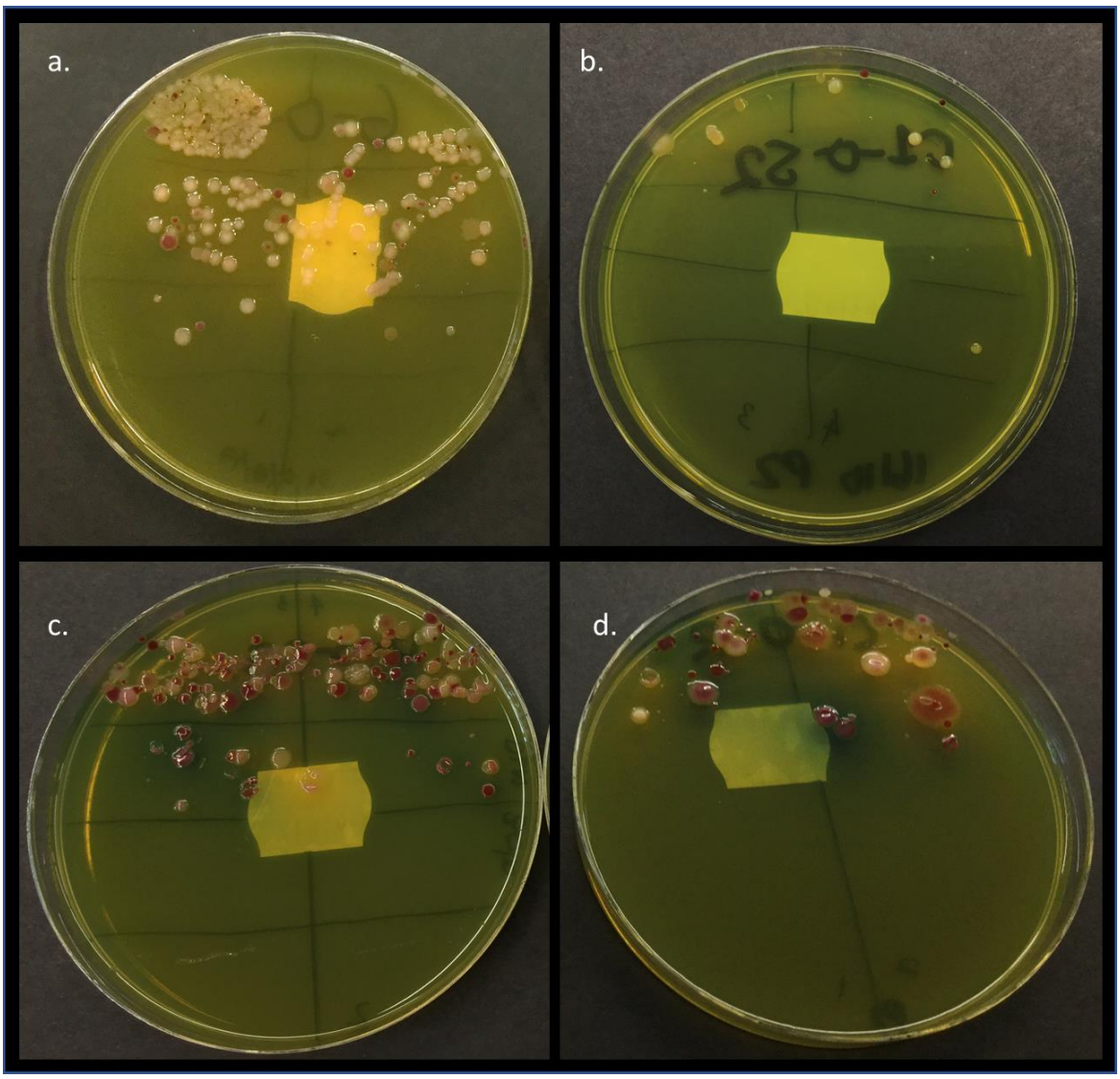

Figure 5. Different hospital wastewater sites had highly different numbers and morphologies of isolated colonies when grown on Tergitol-7 agar
Hospital wastewater samples were serially diluted and plated onto Tergitol-7 agar without any additional antibiotics. 10uL of neat, 10^{-1}, 10^{-2}, 10^{-3} diluted samples were plated in duplicate onto a single plate from each of the following sites: ***a)*** *Site 1 – Radiology/Oncology and* ***b)*** *Site 2 – Residential wing* ***c)*** *Site 3 – Theatre/Outpatient/Emergency Department and* ***d)*** *Reference Site.*

To increase selectivity and to screen for drug resistant isolates, 1mg/L of ciprofloxacin, an antibiotic part of the fluroquinolone family commonly used to treat infections, was supplemented into Tergtitol-7 before pouring and plating. This concentration of ciprofloxacin was chosen to reflect EUCAST's reported ECOFF value of 1mg/L for *P. aeruginosa*. The ciprofloxacin supplemented Tergitol-7 plates (TergC+) were highly selectively, isolating significantly fewer numbers of organisms, compared to Tergitol-7 without antibiotic supplementation as illustrated in Figure 6. This suggested

supplementation of ciprofloxacin into a selective agar such as Tergitol-7 may successfully select resistant organisms.

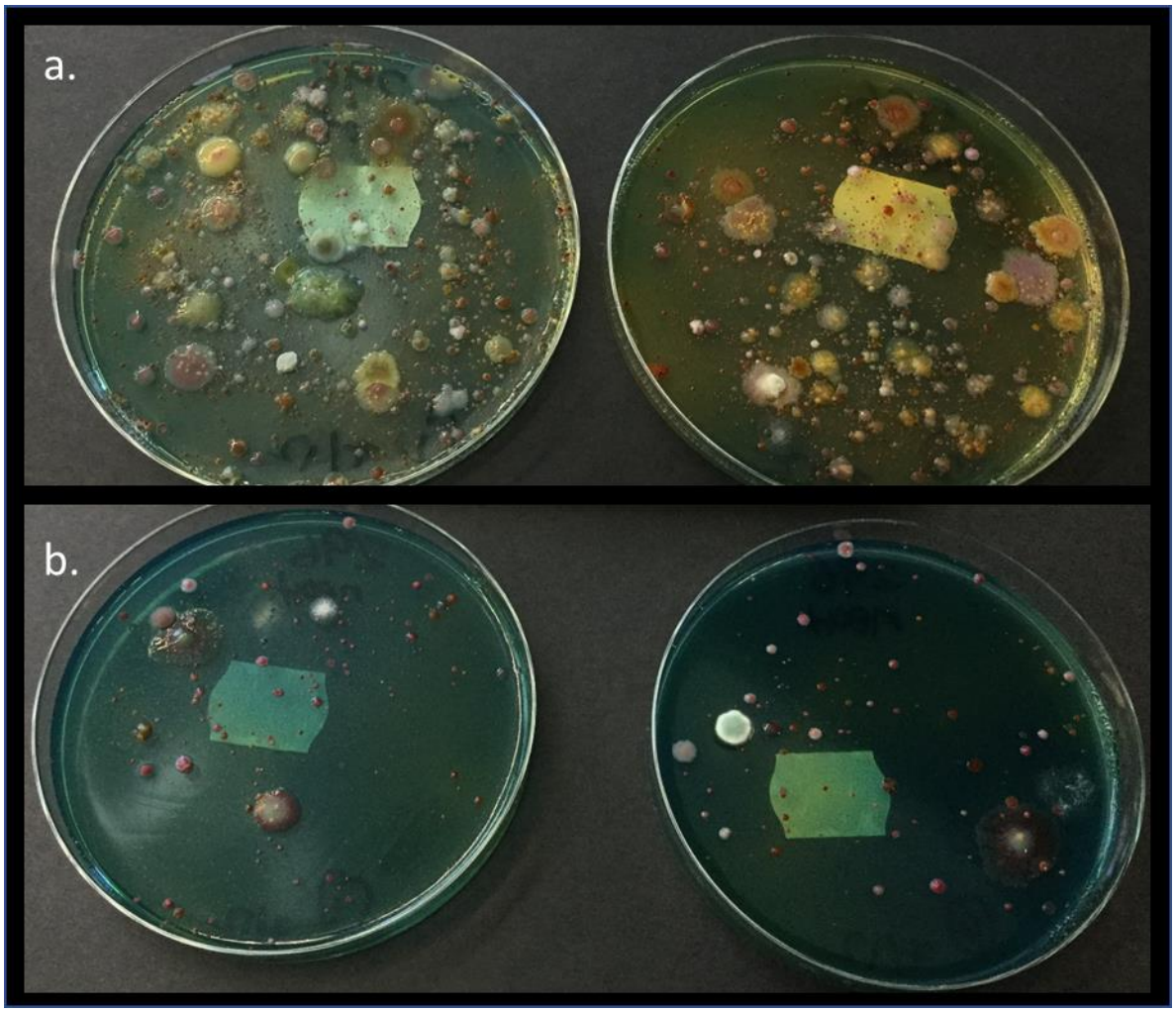

Figure 6. Differences in colony morphologies and numbers seen in Tergitol-7 plates with and without the addition of 1mg/L ciprofloxacin
Tergitol-7 agar was used to isolate organisms from 100uL of neat wastewater samples and further selectivity was achieved with the addition of antibiotics, resulting in distinct differences in numbers and colony types are seen in the above figure. This diagram shows distinct selection when sampled on ***a)*** *Duplicate plates of Tergitol-7 without addition of antibiotics and* ***b)*** *Duplicate plates of Tergitol-7 with addition of 1mg/L ciprofloxacin.*

Amongst all Tergitol-7 plates, the most commonly isolated genera were *Enterobacter spp.* 20.0% (n=110), *Klebsiella spp.* 19.7% (n=108) and *Acinetobacter spp.* 18.4% (n=101) [Figure 7]. The proportion of bacterial genera varied between plates with and without the addition of ciprofloxacin. The proportion of *Klebsiella spp*. were relatively similar amongst all plates; *Klebsiella spp.* represented 38.5% of the organisms on Tergitol-7 plates without any antibiotic (Terg0) and 21.4% on Tergitol-7 plates supplemented with ciprofloxacin (TergC+). *Enterobacter spp*. represented over a fifth of isolates from Terg0 (22.7%) but was found in smaller proportion on TergC+ plates (14.4%). *Raoultella spp.*, a predominantly

environmental organism, represented 8.4% on Tergitol-7 plates without selection compared to TergC+ plates (2.5%).

The "Other" genera [Figure 7] isolated from Tergitol-7 plates included *Acidovorax spp.*, *Brevundimonas spp.*, *Delftia spp.*, *Hafnia spp.*, *Kluyvera spp.*, *Lecleria spp.*, *Lelliottia spp.*, *Ochrobactrum spp.*, *Pantoea spp.*, *Rahnella spp.*, *Wausterseilla spp.*, and *Yersinisa spp*.

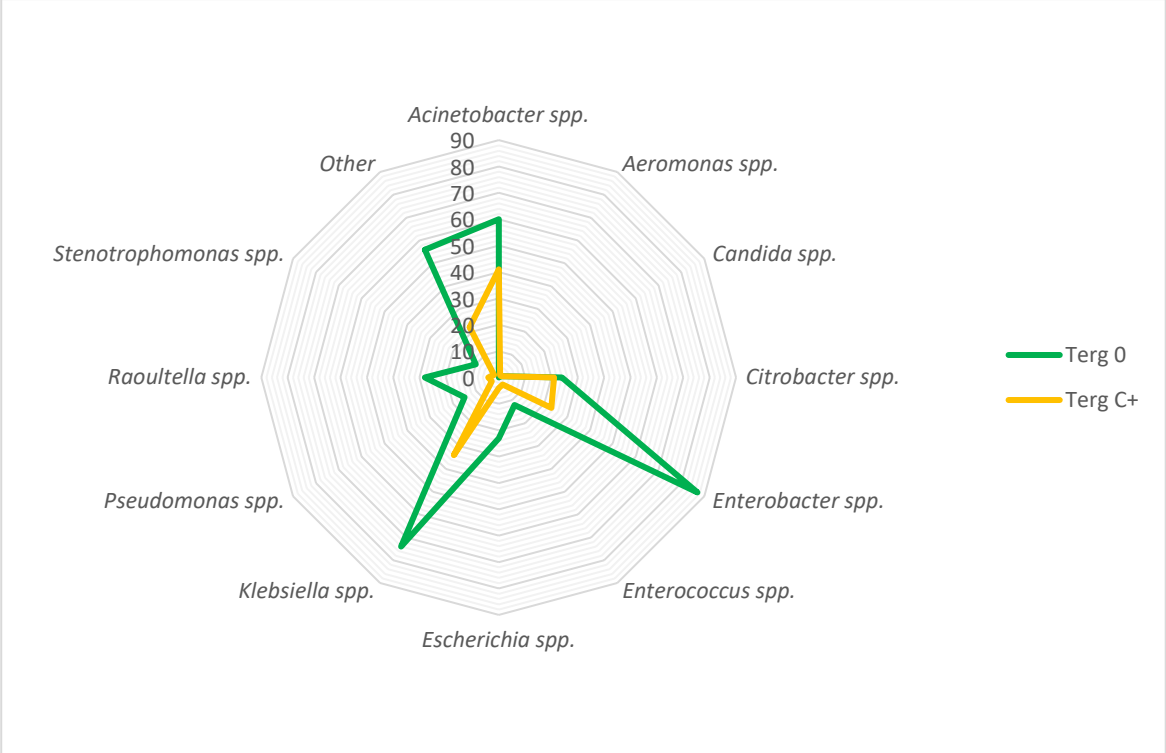

Figure 7. Proportion of genera recovered with Tergitol-7 plates from wastewater of a major metropolitan hospital.
The proportion of different genera isolated on Tergitol-7 plates without antibiotic supplementation (Terg0), (green) compared with Tergitol-7 supplemented with 1 mg/L ciprofloxacin (TergC+) (Yellow). The most commonly isolated genera from Terg0 includes Enterobacter spp. and Klebsiella spp. The most commonly isolated genera from TergC+ includes Acinetobacter spp. and Klebsiella spp.

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3.1.2 Several Pseudomonas subspecies were isolated from Pseudomonas agar

Pseudomonas agar is a selective medium containing cetyltrimethylammonium bromide (cetrimide) for the isolation of *P. aeruginosa* and other species of *Pseudomonas*. Cetrimide acts as a quaternary ammonium compound (acting as a cationic detergent) to inhibit the growth of other bacteria by causing leakage of ions such as nitrogen and phosphorous. *P. aeruginosa* presents as typical bright green colonies as a result of the production of an iron chelator, pyoverdine (yellow in colour) and a virulence factor, pyocyanin (blue). Other

pseudomonads appear buff/cream in colour [Figure 8]. A total of 140 isolates were recovered from all *Pseudomonas* agar plates [Figure 9].

The growth of *Klebsiella spp*., *Providencia spp*., and *Proteus spp.* should be supressed, due to the nalidixic acid in the C-N Supplement. However, even though *Pseudomonas spp.* was the most commonly isolated genera 55% (n=77), *Klebsiella spp.* 9.3% (n=13) was also isolated. A total of 20 different Pseudomonas species were found including: *P. aeruginosa*, *P. asplenii, P. chlororaphis, P. corrugata, P. extremorientalis, P. fluorescens, P. fuscovaginae, P. jessenii, P. koreensis, P. monteilii, P. putida, P. nitroreducens, P. oleovorans, P. plecoglossicida, P. protegens, P. putida, P. synxantha, P. rhodesiae, P. tolaasii, P. veronii*. *P. aeruginosa* isolates varied in colour from light-dark green or clear colonies. Only one green, non-*P. aeruginosa* isolate, *Citrobacter braakii*, was isolated.



Figure 8. Colonies from Pseudomonas Agar plates, without additional antibiotics
Pseudomonas agar without the addition of antibiotics was used to isolate Pseudomonas spp. from hospital wastewater. Pseudomonas spp. colonies from hospital wastewater appeared buff in colour. Hospital wastewater samples were serially diluted and plated onto Tergitol-7 agar without any additional antibiotics. 10uL of neat, 10^{-1}, 10^{-2}, 10^{-3} diluted samples were plated in duplicate onto a single plate as shown here. This figure shows isolates from wastewater from Site 1- Radiology/Oncology.

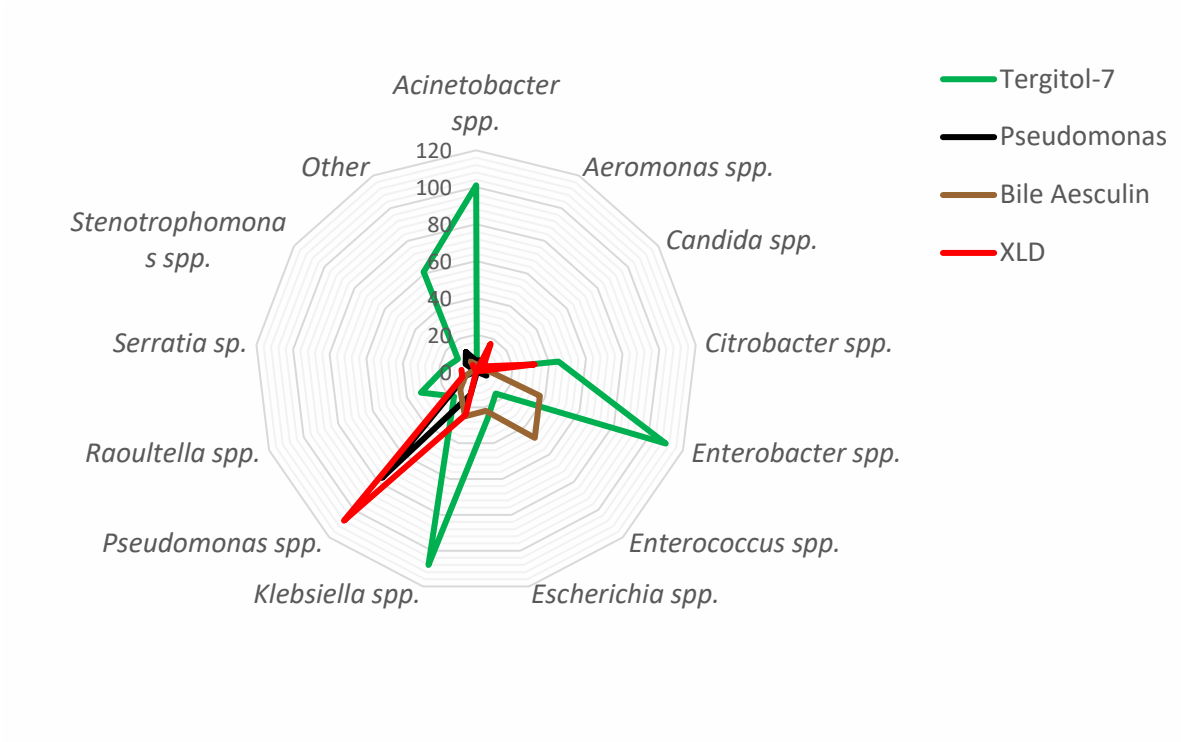

Figure 9. Proportion of genera recovered with various selective plates from wastewater of a major metropolitan hospital.
The proportion of different genera isolated on various selective agar: Tergitol-7 plates (green) with and without antibiotic supplementation, Pseudomonas agar (black) with and without antibiotic supplementation, Bile Aesculin (brown) with vancomycin supplementation and XLD agar (red).

3.1.3 Bile Aesculin recovered E. faecium but not E. faecalis

Gram-positive *Enterococci* were of interest as *Enterococci* are considered a significant public health threat due to their high level of MDR and rates of nosocomial infections [188]. Bile aesculin agar is a differential and selective media used to select for *Enterococci* and group D *Streptococci* based on their ability to hydrolyse aesculin. *Enterococcus* spp. appear as black/grey colonies surrounded by a black precipitate. The presence of oxygall inhibits the growth of other Gram-positive organisms and azide inhibits the growth of Gram-negative bacteria. A total of 170 isolates were recovered from bile aesculin plates. All isolated enterococcus isolates displayed the expected morphology, however, other organisms also shared similar appearances eg. *Acinetobacter*, *Enterobacter*, *Escherichia* and *Klebsiella*, could also appear as grey colonies with a black precipitate. Bile Aesculin plates are most commonly used for isolation from samples far less complex than wastewater and unsurprisingly its use here resulted in the recovery of some Gram-negative species. These Gram-negative organisms had a similar colony morphology to *Enterococcus* and was

not phenotypically distinguishable from *Enterococcus* [Figure 10]. As expected, *E. faecalis*, being a Gram-positive organism and sensitive to the addition of vancomycin, was not isolated from these plates.

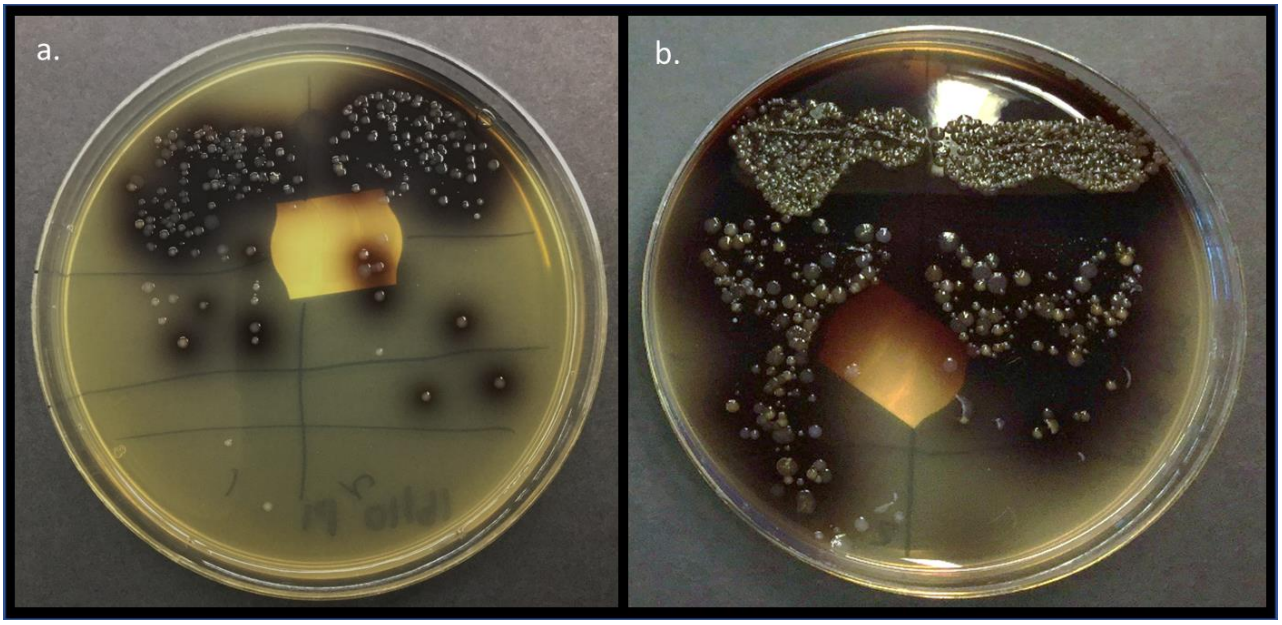

Figure 10. Wastewater from the Residential Wring and Emergency departments showed highly different numbers of organisms when grown on Bile-Aesculin agar supplemented with vancomycin.
Hospital wastewater samples were serially diluted and plated onto Bile-Aesculin agar supplemented with 4mg/L of vancomycin. 10uL of neat, 10^{-1}, 10^{-2}, 10^{-3} diluted samples were plated in duplicate onto a single plate. This figure shows plates prepared from ***a)*** *Site 2 – Residential wing and* ***)*** *Site 3 – Theatre/Emergency. Note the presence of Enterococci and other similar organisms seen as cream colonies with black precipitate and the presence of other organisms seen as cream colonies without the presence of lace precipitate.*

3.1.4 XLD agar cannot sufficiently distinguish between isolates of complex wastewater sample

A total of 206 isolates were recovered from XLD plates but it is important to note XLD plates were only used in the initial isolation process due its similarities and lack of broad selection of organisms of interest when compared to Tergitol-7 agar. XLD is clinically used to isolate *Salmonella* and *Shigella species*, as well as other coliforms from faecal specimens but within this project, was unable to be a sufficient medium for a more complex environment such as wastewater. Figure 9, Table 4. and Table 5 illustrate that XLD plates isolated a smaller selection of organisms in comparison to Tergitol-7 agar. Although XLD has very useful applications as a selective and differential medium for clinical specimens, it was unable to isolate significant numbers of organisms from wastewater. Thus, results show

the difficulties of using established clinically accepted agar for differential isolate of complex, ecological samples. The use of XLD plates for isolation of organisms from a complex sample was not as effective as using Tergitol-7. Clinically, XLD is used for the presumptive isolation of *Salmonella spp*. however only one *Salmonella* was found. If future studies wanted to isolate *Salmonella spp*., the use of enrichment techniques, such as Rappaport-Vassiliads broths, would be necessary for obtaining *Salmonella spp*. Furthermore, in comparison to Tergtiol-7, colonies growing on XLD were difficult to distinguish, appearing only as clear colonies with varied sizes and borders.

Table 5. Expected and observed morphologies on XLD agar without selective antibiotics.

Organism	Expected morphology	Observed morphology
K. pneumoniae	Yellow, opaque	Round/irregular, yellow, mucoidal
S. flexneri	Red	None found
S. typhimurium	Red with back centres	Red with back centre
E. coli	Inhibited	None found
S. faecalis	Red with blue zone	None found
P. aeruginosa	Red/pink, flat	Pink/clear

3.2 Investigation of the microecological populations in wastewater

This project successfully isolated a large variety of organisms of interest for further investigation. This included *Pseudomonas spp. Klebsiella spp.*, *Citrobacter spp.*, *Enterobacter spp*. and this will be discussed further in this section. Several organisms were not identifiable by the MALDI-TOF, an expected limitation of using a MALDI-TOF with a predominantly clinical database for analysing environmental samples. These unidentifiable organisms were not further investigated due to time restraints of the project. As expected, no Gram-positive organisms were isolated from plates other than Bile Aesculin.

3.2.1 Pseudomonas spp. were the most frequently isolated family

Of all bacterial species isolated, the *Pseudomonas* genus was the most frequently isolated, representing 20.3% (n=216) of isolates. In total, 30 different *pseudomonas* species were found, also representing the most diverse family isolated. The most prevalent species were *P. aeruginosa* 17.6% (n=38) and *P. putida* 19.9% (n=43). Followed closely by *P. koreensis* 9.3% (n=20) and *P. tolaasii* 6.0% (n=13) [Figure 11]. The *Pseudomonas* species were predominantly isolated from Pseudomonas plates but were also found on Tergitol-7 and Bile Aesculin.

Data from this section formed part of a subsequent paper: "Amsalu, A.; Sapula, S.A.; De Barros Lopes, M.; Hart, B.J.; **Nguyen, A.H.**; Drigo, B.; Turnidge, J.; Leong, L.E.; Venter, H. Efflux Pump-Driven Antibiotic and Biocide Cross-Resistance in *Pseudomonas aeruginosa* Isolated from Different Ecological Niches: A Case Study in the Development of Multidrug Resistance in Environmental Hotspots. *Microorganisms* **2020**, *8*, 1647 [Appendix 1]. Within this paper wastewater *P. aeruginosa* isolates had a much higher proportion of multidrug resistance compared to clinical and veterinary samples [17].

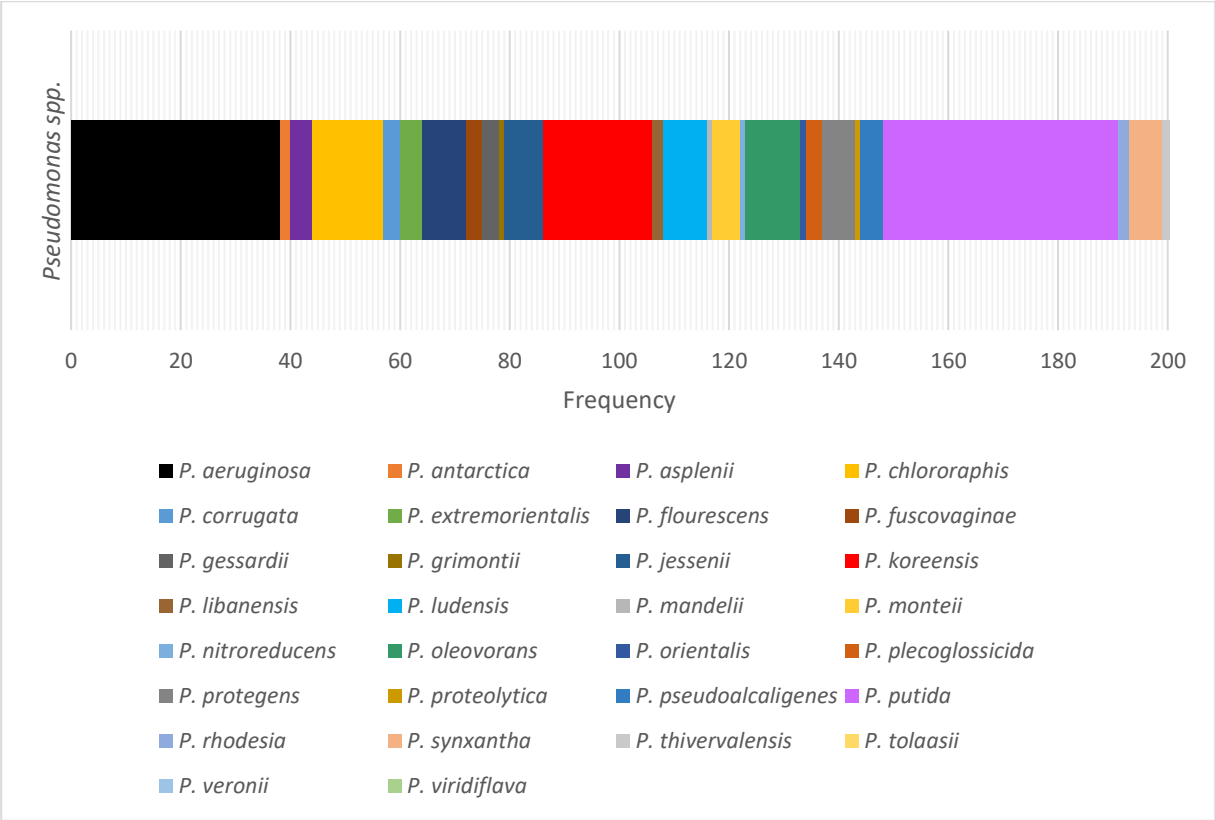

Figure 11. A large diversity of Pseudomonas spp. isolated from 4 different sites within a major hospital.
This figure shows the diversity of *Pseudomonas spp*. recovered from hospital wastewater across 4 sites: Radiology/Oncology, Residential, Theatre/Emergency Department and a Reference site when isolated on Tergitol-7 agar, Tergitol-7 agar with 1mg/L ciprofloxacin, Pseudomonas Agar, Bile-Aesculin with 4mg/L Vancomycin and XLD agar. In total, 30 different *Pseudomonas* species were isolated (n=200). The most prevalent species was *P. putida* (n=43) followed by *P. aeruginosa* (n=38).

3.2.2 Klebsiella spp. were the second most prevalent family isolated

The *Klebsiella* genus were the second most prevalent present in wastewater, representing 16.0% (n= 170) of all isolates found. Only 3 different species were isolated, *K. variicola* 3.5% (n=6)), *K. oxytoca* 72.9% (n=124) and *K. pneumoniae* 23.5% (n=40). *K. oxytoca* were the most frequently isolated species amongst all genera, representing 11.6% (n=124) of all isolated organisms. Most of the *Klebsiella spp.*, 63.5% (n=108) were recovered from Tergitol-7 plates: Terg0, 43.5% (n=74), TergC+, 20.0% (n=34). BileV+ recovered 14.7% (n=25) of the *Klebsiella spp*.

It was interesting to note the significant prevalence of *K. oxytoca* species, an environmental species noted for being an emerging, opportunistic pathogen. Due to its emerging dangers in human health, investigations with *K. oxytoca* were explored further in Chapter 4.

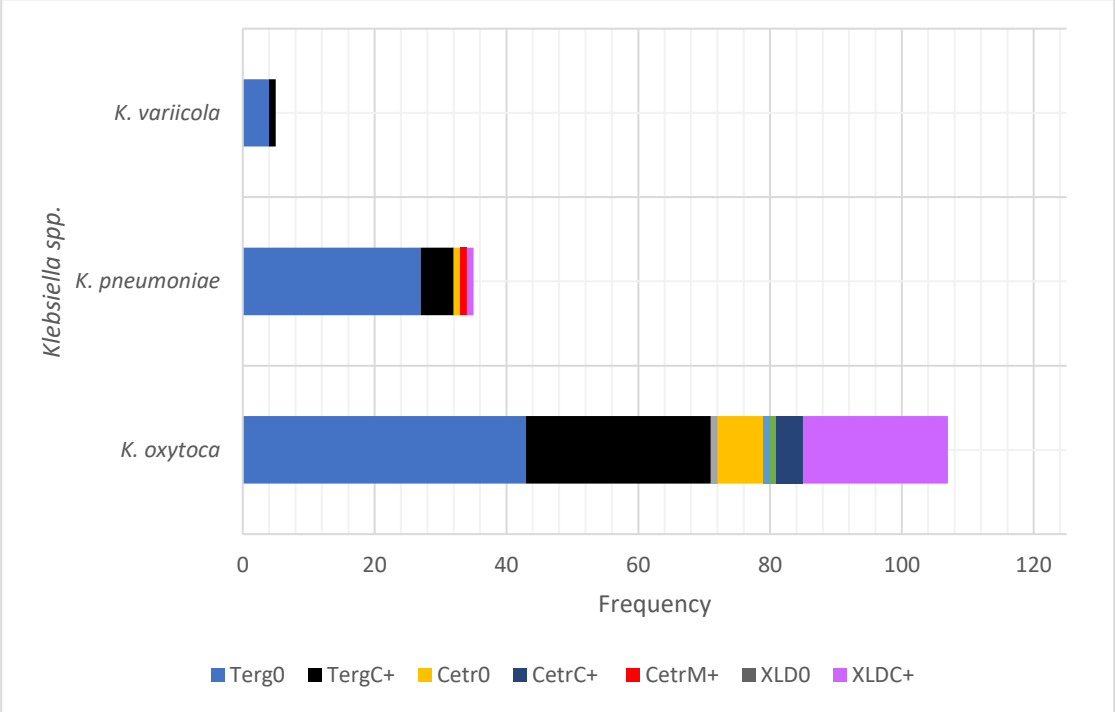

Figure 12. Selective media isolated 3 species of Klebsiella amongst 4 different wastewater sites within a major hospital

This figure shows the frequency of isolated *Klebsiella spp*. recovered from hospital wastewater across 4 sites: Hospital Outpatients, Emergency Department, Oncology and a Reference site, when isolated on different selective medium: Tergitol-7 without antibiotics, Tergitol-7 supplemented with 1mg/L ciprofloxacin, Pseudomonas agar, XLD without antibiotics, XLD with 1mg/L ciprofloxacin, XLD with Meropenem and Bile Aesculin with vancomycin. In total, 3 different *Klebsiella* species were isolated (n=170). The most prevalent species was *K. oxytoca* (n=124), followed by *K. pneumoniae* (n=40) and finally *K. variicola (n=6).*

3.2.3 Citrobacter spp. consisted of 5 different species

The *Citrobacter* genus represented 8.3% (n=88) of all isolates found andconsisted of 5 different species: *C. amalonaticus* (4.3%), *C. braakii* (29.0%), *C. farmeri* (3.2%), *C. freundii* (61.73) and *C. gillenii* (2.2%) [Figure 13; Figure 14].

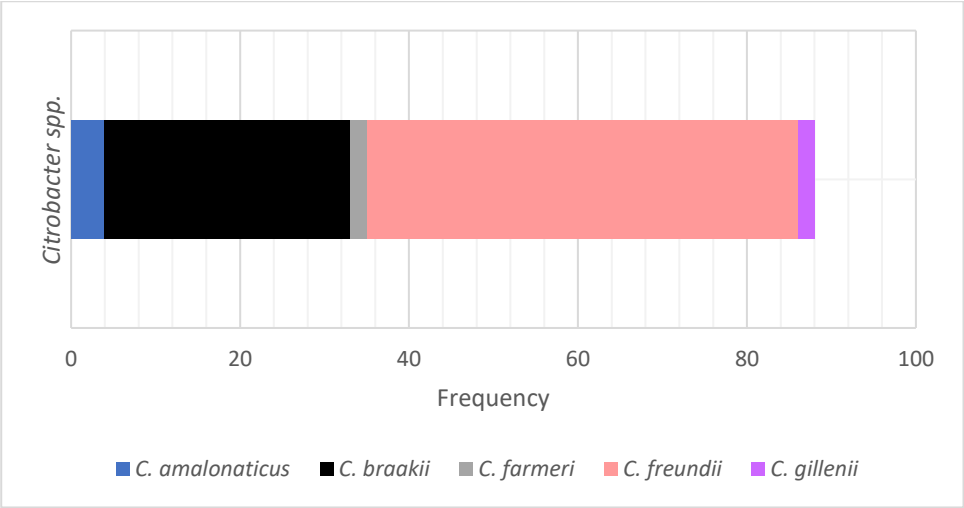

Figure 13. Selective media isolated 5 species of Citrobacter spp. amongst 4 different wastewater sites within a major hospital

This figure shows the frequency of isolated *Citrobacter spp*. recovered from hospital wastewater across 4 sites: Hospital Outpatients, Emergency Department, Oncology and a Reference site, when isolated on different selective medium: Tergitol-7 without antibiotics, Tergitol-7 supplemented with 1mg/L ciprofloxacin, Pseudomonas agar, XLD without antibiotics, XLD with 1mg/L ciprofloxacin, XLD with Meropenem and Bile Aesculin with vancomycin. A total of 5 different *Citrobacter* species were isolated (n=88). The most prevalent species was *C. freundii* (n=51) followed by *C. braakii* (n=29).

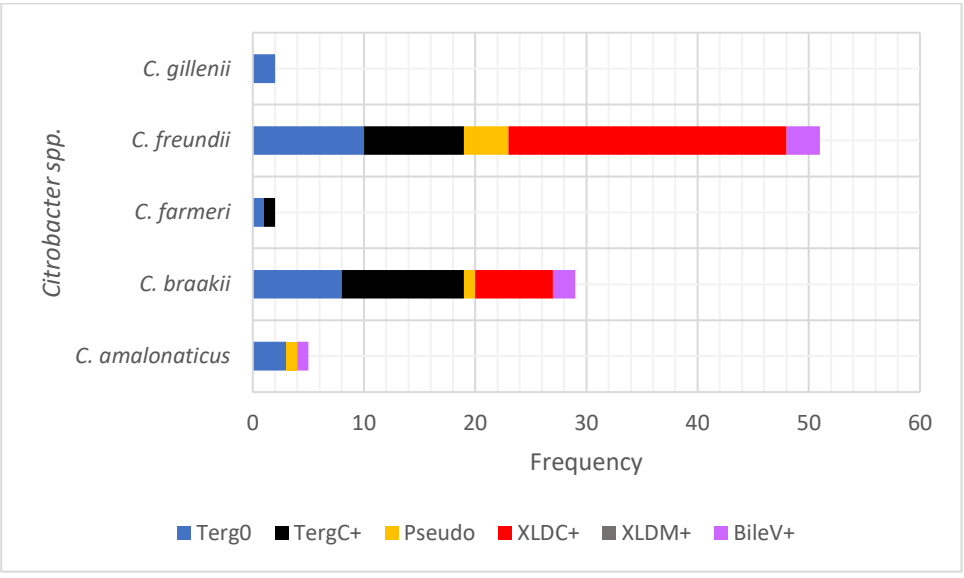

Figure 14. Selective media isolated 5 species of Citrobacter spp. amongst 4 different wastewater sites within a major hospital

This figure shows the frequency of isolated *Citrobacter spp*. recovered from hospital wastewater across 4 sites: Hospital Outpatients, Emergency Department, Oncology and a Reference site, when isolated on different selective medium: Tergitol-7 without antibiotics, Tergitol-7 supplemented with 1mg/L ciprofloxacin, Pseudomonas agar, XLD without antibiotics, XLD with 1mg/L ciprofloxacin, XLD with Meropenem and Bile Aesculin with vancomycin. A total of 5 different *Citrobacter* species were isolated (n=88). The most prevalent species was *C. freundii* (n=51) followed by *C. braakii* (n=29).

This study has revealed a high prevalence of *Citrobacter spp*. with phenotypic resistance against ciprofloxacin. *Citrobacter spp.* is an increasingly emerging pathogen and thus, is an organism worth being investigated further in follow up studies.

3.2.4 Three different species of Enterobacter were isolated

The *Enterobacter* genus represented 14.4% (n=153) of all isolates recovered and consisted of 3 different species as presumptively assigned by MALDI-TOFF: *E. asburiae* 45.6% (n=70), *E. cloacae* 35.9% (n=53) and *E. kobei* 18.3% (n=28). *E. cloacae* represented 3.6% (n=55) of all isolated organisms [Figure 15]. *E. asburiae, E. kobei* and *E. cloacae* represents 3 of the 6 organisms within the *Enterobacter Cloacae Complex* , a classification of *Enterobacter* considered clinically significant [187]. Most *Enterobacter* (71%) were recovered from Tergitol-7 plates [Figure 9].

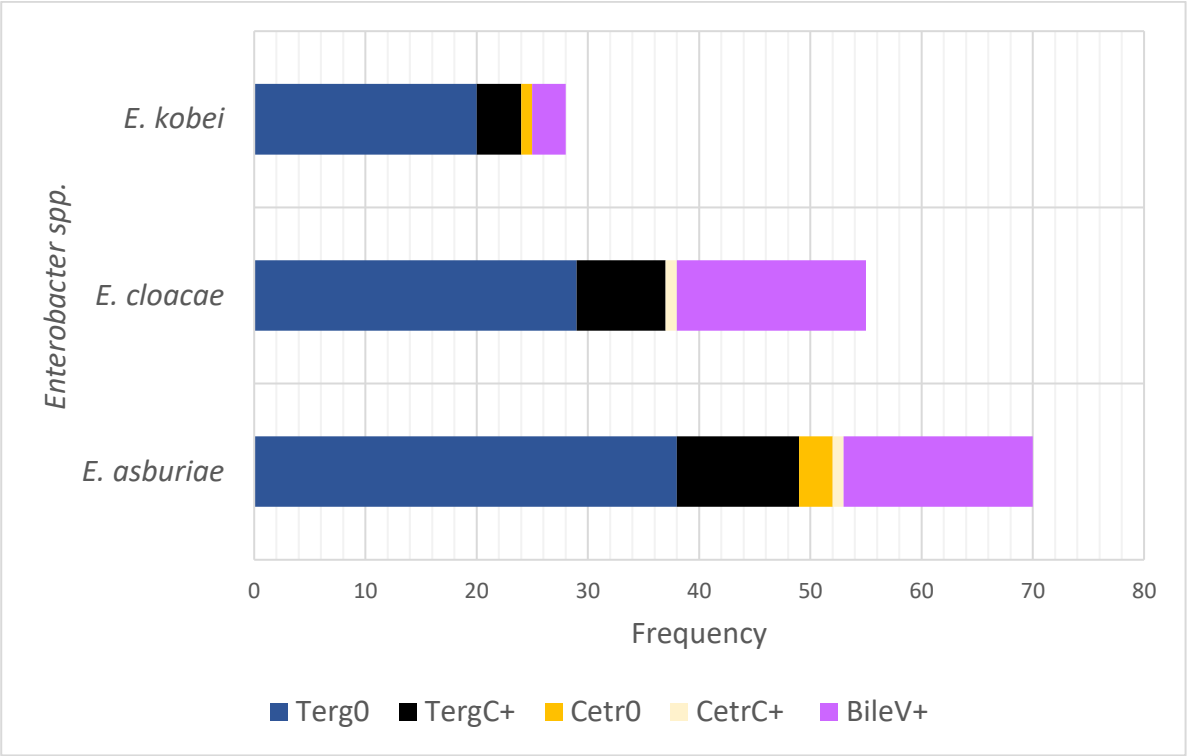

Figure 15. Selective media isolated 3 species of Enterobacter spp. amongst 4 different wastewater sites within a major hospital.

This figure shows the frequency of 3 isolated *Enterobacter spp*. from the *Enterobacter Complex*, recovered from hospital wastewater across 4 sites: Hospital Outpatients, Emergency Department, Oncology and a Reference site, when isolated on different selective medium: Tergitol-7 without antibiotics, Tergitol-7 supplemented with 1mg/L ciprofloxacin, Pseudomonas agar and Bile Aesculin with vancomycin. A total of 3 different *Enterobacter* species were isolated (n=153). The most prevalent species was *E. asburiae* (n=70) followed by *E. cloacae* (n=55) and *E. kobei* (n=28).

Enterobacter spp. are a prevalent pathogenic species of interest, known to be carriers of many antibiotic resistance genes. The *Enterobacter sp*p. isolated in this study formed the basis of a follow up study exploring the epidemiology of *Enterobacte*r in hospital wastewater. The project is currently in progress by Naomi Siderius (PhD candidate).

3.2.5 A. towneri was the most common of nine different species of Acinetobacter isolated

Acinetobacter genus represented 10.4% (n=111) of all isolates found and consisted of 9 different species: the most prevalent species were *A. towneri* 38.7% (n=43), followed by *A. johnsonii* 20.7% (n=23) and *A. junii* 15.3%(n=17).

As seen from Figure 16, *A. towneri* are a highly prevalent, environmental species. Early work suggests they may commonly be ciprofloxacin resistance and isolates have formed a part of unpublished results revealing a new unknown species of Acinetobacter through whole genome sequencing methods.

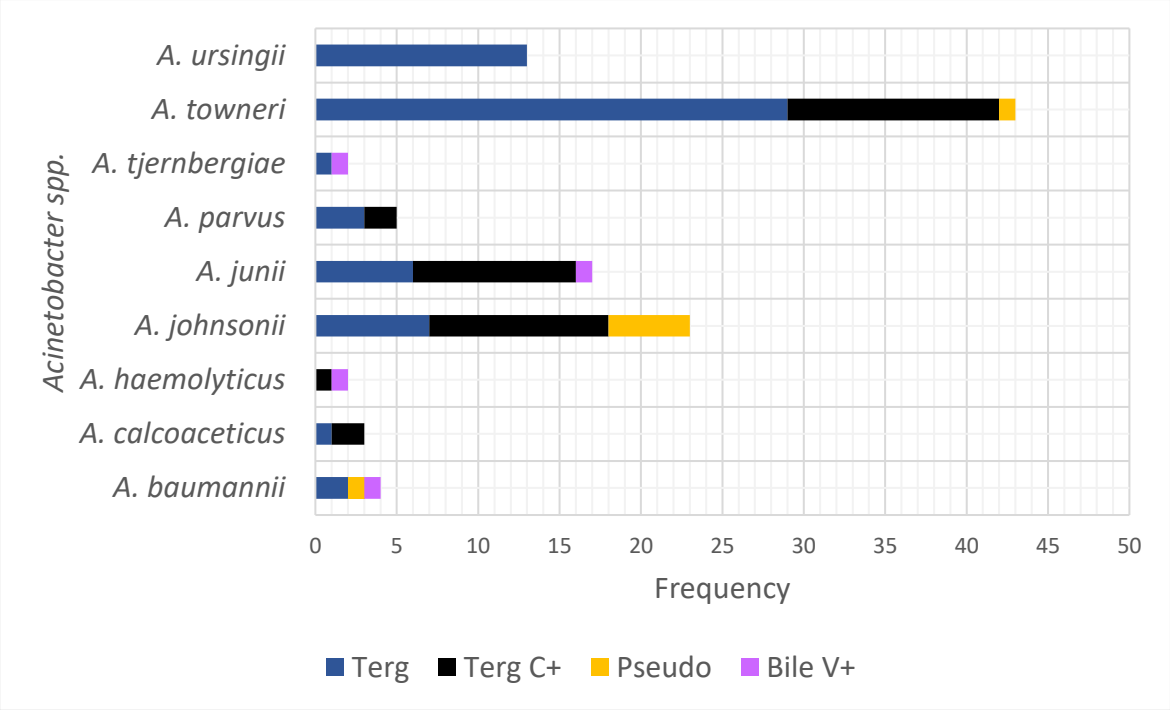

Figure 16. Selective media isolated 9 species of Acinetobacter spp. amongst 4 different wastewater sites within a major hospital.

This figure shows the frequency of isolated *Acinetobacter spp*. recovered from hospital wastewater across 4 sites: Hospital Outpatients, Emergency Department, Oncology, and a Reference site, when isolated on different selective medium: Tergitol-7 without antibiotics, Tergitol-7 supplemented with 1mg/L ciprofloxacin, *Pseudomonas* agar and Bile Aesculin with vancomycin. A total of 9 different *Acinetobacter* species were isolated (n=111). The most prevalent species was *A. towneri* (n=43) followed by *A. johnsonii* (n=23) and *A. junii* (n=17).

3.2.6 Two species of vancomycin resistant Enterococci were isolated

The *Enterococcus* genus represented 5.9% (n=63) of all isolates found and consisted of 2 different species: *E. casseliflavus* 1.6% (n=1) and *E. faecium* 98.4% (n=62). No *E. faecalis* isolates were found. Of the *Enterococcus spp*. found, 76.2% (n=48) were isolated from BileV+ plates [Figure 17]. As expected, no *E. faecalis* were isolated due to the selection with vancomycin. *E. faecium* is considered less prevalent than *E. faecalis* and can be highly

vancomycin resistant, comparatively, *E. faecalis* vary in their level of vancomycin resistance [188]. Further epidemiological studies on the vancomycin resistant *E. faecium* from hospital wastewater are currently being undertaken by Belayenash Tachebele (PhD candidate).

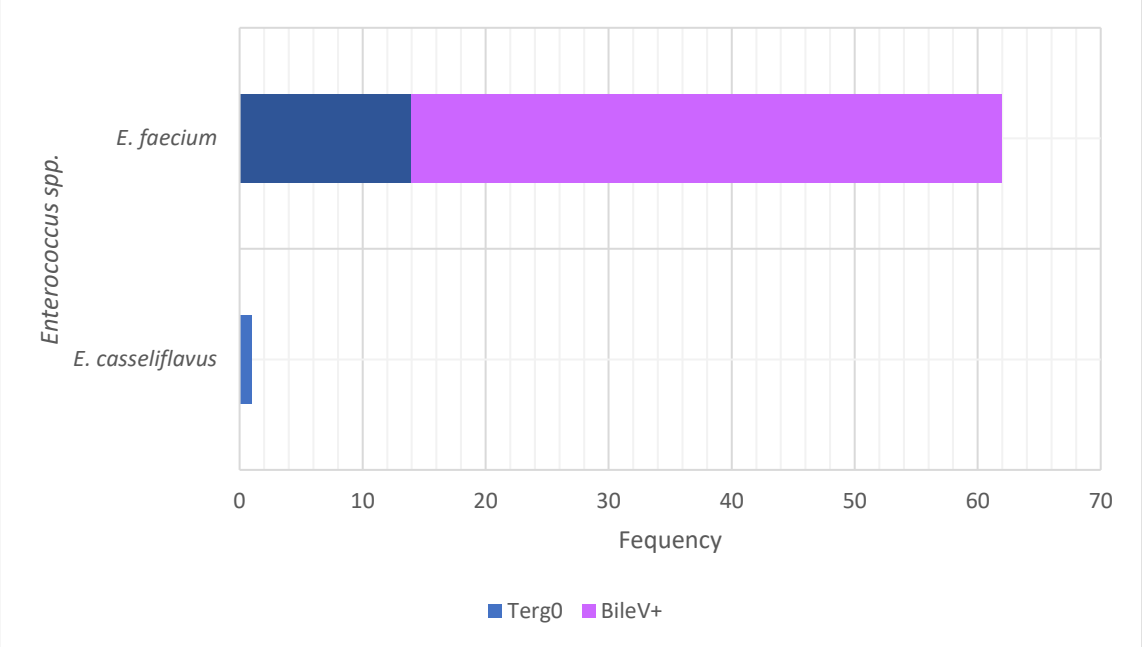

Figure 17. Selective media isolated 2 species of Enterococcus spp. amongst 4 different wastewater sites within a major hospital.
This figure shows the frequency of isolated *Enterococcus spp*. recovered from hospital wastewater across 4 sites: Hospital Outpatients, Emergency Department, Oncology, and a Reference site, when isolated from different selective medium: Tergitol-7 without antibiotics and Bile aesculin with vancomycin. In total, 2 different *Enterococci species* were isolated (n=63). The most prevalent species was *E. faecium* (n=62), followed by *E. casseliflavus* (n=1). *E. faecalis* was not isolated.

3.2.7 Other notable species

Results showed the *Escherichia* genus represented 4.8% (n= 52) of all isolated organisms. Isolated *Escherichia* consisted of 3 species, *E. hermannii* (21.2%), *E. vulneris* (7.7%) and *E. coli* (71.2%). *E. coli* represented 3.4% (n=37) of all isolated organisms. *Escherichia spp.* were not an organism of focus within this project as it is not as resilient in wastewater compared to some of the other Enterobacerialis e.g. the Enterobacter cloacae complex.

Other notable bacterial species isolated include *Aeromonas* spp. (n=25), *Wauterseilla* spp. (n=18), *Acidovorax* spp. (n=9), *Chryseobacterium* spp. (n=7), *Pantoea* spp. (n=7) amongst others. These species are predominantly environmental organisms (*Wauterseilla spp.*,

Acidovorax spp.) but can be opportunistic pathogens (*Aeromonas spp., Chryseobacterium spp., Pantoea spp.*) and should be studied in future projects.

Only one non-bacterial species was unintentionally isolated; four different *Candida* species representing 0.75% (n=8) of all isolates*; C. glabrata, C. tropicalis, C. albicans* and *C. parasilosi. C. glabrata* was isolated from BileV+; all other *Candida spp*. were isolated from XLD agar plates.

3.2.8 Summary of phenotypically isolated organisms

The initial phenotypic isolation found a total of 1065 isolates amongst 102 different species. A mix of pathogenic and environmental organisms were isolated, and it is noteworthy each site had a very different proportion of bacterial species. This is represented in Figure 18. The most commonly isolated organisms belonged to the *Pseudomonas* genus, followed by *Klebsiella spp.* and *Enterobacter spp*. [Figure 18]. Each species was distributed differently between each tested wastewater site, the noteworthy species with the highest prevalence from each site includes: 52% of *Acinetobacter spp*. found in Reference Site, 52% of *Citrobacter spp.* in Site 1, 38% of *Enterobacter spp*. in Site 3, 37% of *Klebsiella spp*. isolated in Site 1, 32% and 33% of *Pseudomonas spp*. in Site 2 and Site 3 respectively [Figure 18].

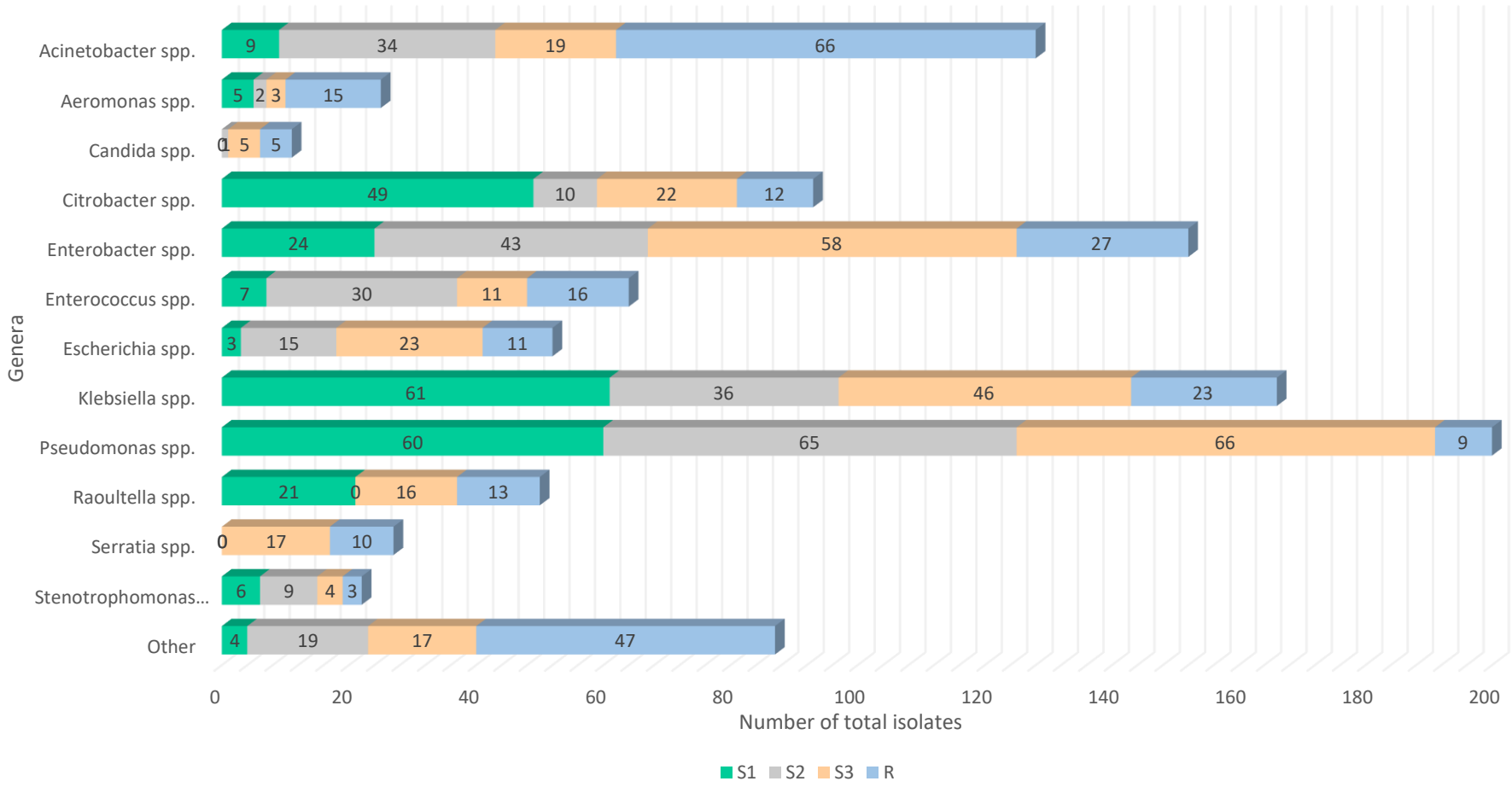

Figure 18. The distribution of the most commonly isolated bacterial genera amongst 4 different wastewater sites within a major hospital.
This figure shows the distribution of the most commonly isolated bacterial genera different wastewater sites across a major hospital site: S1- Radiology/Oncology, S2- Residential Department, S3- Theatre/Emergency Department, and R- Reference site, when isolated on different selective medium: Tergitol-7 without antibiotics, Tergitol-7 supplemented with 1mg/L Ciprofloxacin, *Pseudomonas* agar and Bile Aesculin with vancomycin. The most prevalent genera isolated from at each site were *S1: Klebsiella spp. & Pseudomonas spp., S2: Pseudomonas spp., S3: Pseudomonas spp., R: Acinetobacter spp.*

3.2.9 16s profiling revealed 309 bacterial genera and showed clear differences between population composition of reference site and hospital wastewater sites

To further understand the overall microbial ecology in the wastewater, 16S sequencing was also performed and results were coupled with resistance profiling from Chapter 3.1. Within the 4 wastewater samples, bacterial 16s profiling was able to identify a total of 309 different genera, across 65 orders, 32 classes and 18 phyla [Figure 19]. The most abundant taxonomic units belonged to the phyla *Proteobacteria* or *Firmicutes* followed by *Actinobacteria* or *Bacteroidetes*. The *Firmicutes* phyla include *Enterococcus spp.*, *Proteobacteria include Escherichia spp., Shigella spp., Salmonella spp*., *Actinobacteria* includes *Myobacterium spp*., *Nocardia spp*. It is interesting to observe proportions of taxonomic units varied greatly between the different sites [Figure 19]. For instance, the Reference site had 49035 different genera, the least diverse of the tested sites. The major phyla identified from the Reference site were 66.90% *Firmicutes,* 26.28% *Proteobacteria*, 3.67% *Actinobacteria*. Site 2 contained 32050 different genera, representing the least diverse site within the three hospital wastewater sites. A total of 50601 bacterial genera were identified within Site 1, representing the second most ecologically diverse site. Note the differences in population abundance where Site 1 had only 36.14% *Firmicutes*; half the number found in the Reference site, 53.94% were *Proteobacteria*; double the number found in Reference site. Site 3 had the highest level of diversity with 63131 genera identified, this was 30% more diverse than the Reference site. Comparing the bacterial families of interest across Sites 1,2,3, and the Reference site, *Enterobacterales* was found in different proportions: 7.32%, 0.47%, 2.68% and 4.11% as was *Pseudomonadaceae* 0.72%, 0.26%, 1.95% and 2.16% for Sites 1, 2, 3 and the Reference site respectively. Site 3 had the highest level of diversity in comparison to the other sites. 16s sequencing illustrated a distinction between the ecological niches at each wastewater site, confirming the same observation noted from the phenotypic isolation.

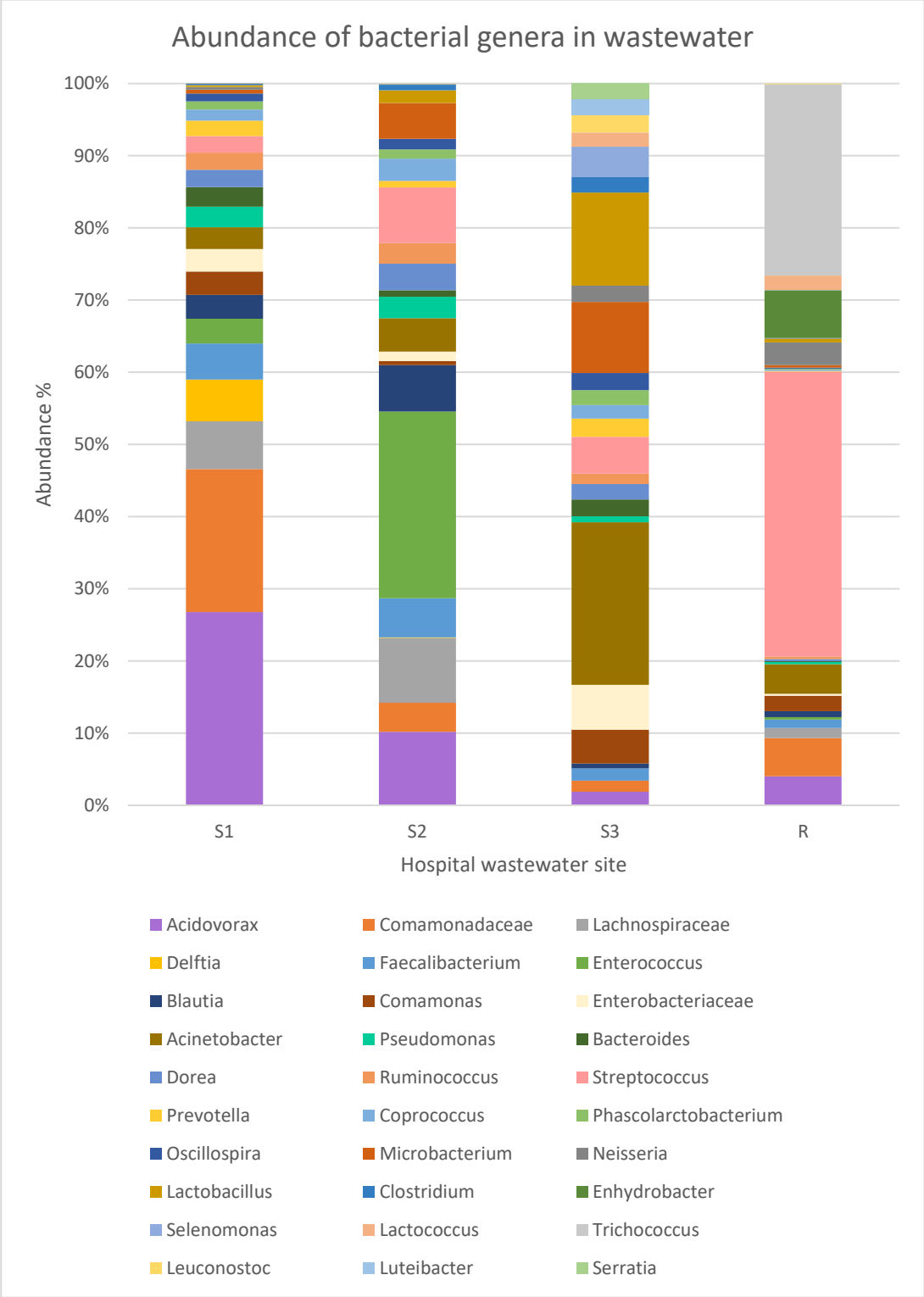

Figure 19. Population profiling of the four hospital wastewater sites using 16s sequencing.

Legend shows the abundance of the top 30 bacterial genera. Site 1 had 50601 genera, Site 2 had 32050, Site 3 had 63131 and reference site had 49035.

3.3 Antibiotic susceptibility testing

To gain an idea of AMR and incidence of MDR, MICs were performed across six species: *C. freundii* (25), *E. cloacae* (25), *E. coli* (35), *K. pneumoniae* (34), *K. oxytoca* (88). Susceptibility testing was not performed for *P. aeruginosa* as this formed part of another project by Anteneh Amsalu (PhD) [Appendix 1]. Overall, MDR organisms were prevalent amongst all tested species with 34% (n=75) being resistant to 3 or more classes of antibiotics.

The antimicrobial susceptibility patterns of 212 isolates were studied using a panel of up to 17 antimicrobials. As the intention was to isolate MDR organisms, a high starting concentration was used to screen isolates. Further plates were performed to find exact MICs for isolates of interest. Isolate MICs were compared to the MICs from the EUCAST breakpoints and this determined whether an isolate was deemed as sensitive or resistant against an antibiotic. Biocide resistance was explored, however, as there exists no official breakpoint data, values MIC values obtained from this paper were compared to an existing paper by Morrissey [91]. It is noteworthy that this current study gathered a smaller population in comparison to Morrissey and as such, may have contributed to different ECOFF values.

3.3.1 C. freundii were multidrug resistant and had intrinsic resistance against triclosan

A total of 25 *C. freundii* isolates were tested for resistance against 7 antibiotics across 5 antibiotic classes: cephalosporins, quinolones, carbapenems, aminoglycosides and polymyxin. 76% (n=19) of isolates were resistant against both cephalosporins [Appendix 2]. All isolates were sensitive against meropenem and colistin and an alarming 84% (n=21) were MDR [Table 6]. Taking into consideration *C. freundii* was isolated from plates other than just the Tergitol-7 C+ (Terg0, Pseudomonas and Bile V+), it is interesting to note *C. freundii* displayed very high resistance against ciprofloxacin.

Table 6. Antibiotic susceptibility profile of 26 C. freundii isolates.

		Number of *C. freundii* isolates with MIC (mg/L)									
Antibiotic	**Site**	0.125	0.25	0.5	1	2	4	8	16	32	>64
FEP	**1**							1	1	2	7
	2	1	2	4	.	1	0	0	0	0	1
	3	.	1	0	0	0	0	0	0	1	1
	4	1	1								
CAZ	**1**									1	10
	2							1	2	2	4
	3			1	2	0	0	0	0	1	
	4			1	0	0	0	0	0	0	1
CIP	**1**						4	4	2	0	1
	2	1	0	0	5	1	0	0	0	1	1
	3			1	0	1	0	0	0	1	
	4		2								
LEV	**1**				5	4	2				
	2	2	0	0	2	2	0	1	0	1	.
	3		2	1							
	4		1	0	1						
GEN	**1**								1	1	9
	2								.	1	8
	3								1	1	1
	4			1	0	0	0	0	0	0	1
TOB	**1**						1	1	5	4	
	2				1	1	1	0	2	2	2
	3						1	0	1		
	4		1	1							
MER	**1**	5	6								
	2	7	1								
	3	3									
	4	2									
COL	**1**	8	3								
	2	7	1								
	3	1	0	1							
	4	2									

The minimum inhibitory concentrations were determined for C. freundii isolates isolated from wastewater from four different sites at one hospital: Site 1: Radiology/Oncology; Site 2: Residential Site 3: Theatre/Emergency/Outpatient; and Site 4: Reference site. Vertical black lines indicate EUCAST ECOFF values [118]. Eight different antibiotics: cefepime (FEP), ceftazidime (CAZ), ciprofloxacin (CIP), levofloxacin (LEV), gentamicin (GEN), tobramycin (TOB), meropenem (MER), colistin (COL).

To our knowledge no ECOFF values have been previously determined for *C. freundii* against chlorhexidine, triclosan, benzalkonium chloride or $AgNO_3$. From this study, the ECOFFFinder determined the *C. freundii's* 95% ECOFF as follows: chlorhexidine 64 mg/L,

benzalkonium chloride 32 mg/L and $AgNO_3$ 64 mg/L [Figure 20]. All *C. freundii* isolates had MICs ≥64mg/L against triclosan [Table 7] possibly indicating intrinsic resistance exists within the species. As such, the ECOFF value was unable to be determined. ECOFFFinder visualised three populations of MICs for $AgNO_3$. The fit modelled the ECOFF at 64 mg/L, with the lowest ECOFF at 4 mg/L. This was a similar pattern recently found in *Pseudomonas spp.* [17].

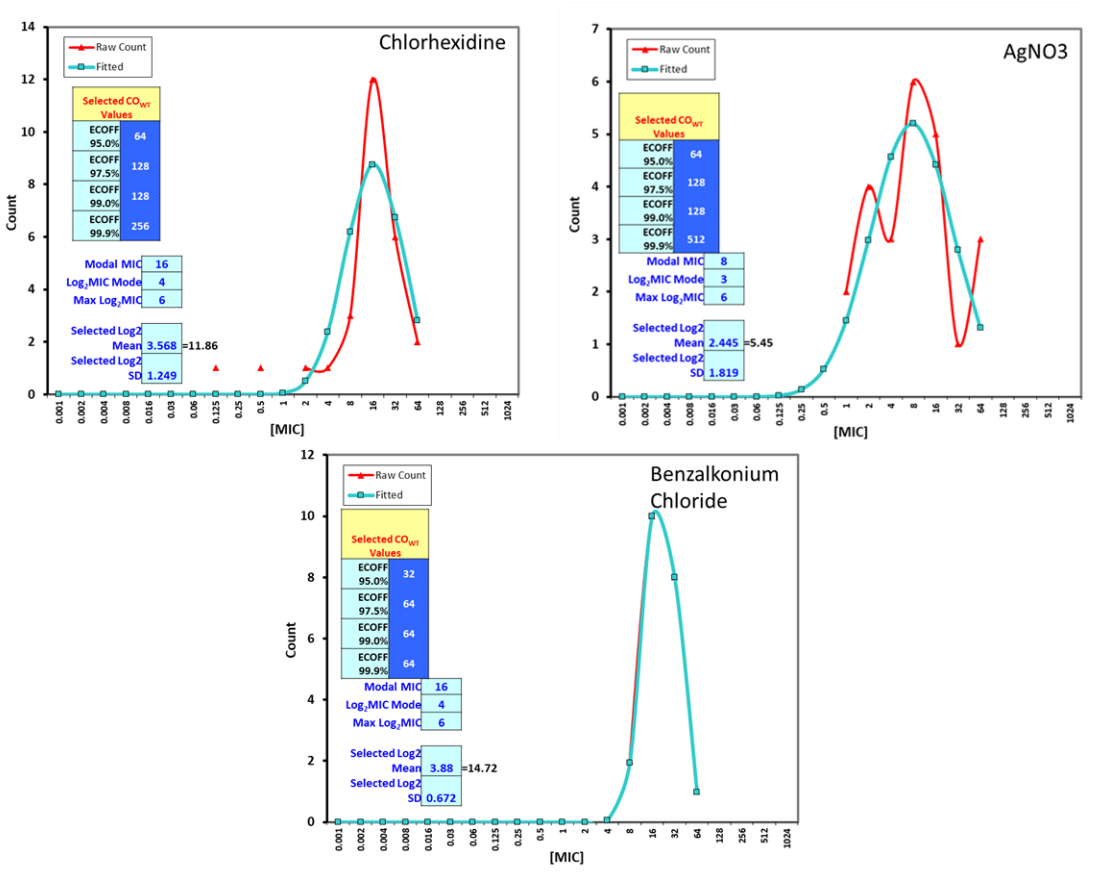

Figure 20. ECOFF values for *C. freundii* against common biocides
The ECOFF values for chlorhexidine, triclosan, benzalkonium and $AgNO_3$ were found (with 95% cut-off) values, using the ECOFFFinderXL2010.xlsm tool (https://clsi.org/meetings/microbiology/ecoffinder/). The 95% ECOFF for *C. freundii* was 64mg/L against chlorhexidine, 32mg/L against benzalkonium chloride and 64mg/L against $AgNO_3$. The ECOFF value for triclosan was not able to be determined as the MIC was consistently≥64mg/L suggesting intrinsic resistance for this species.

Table 7. Biocide susceptibility profile of 26 C. freundii isolates.

		Number of *C. freundii* isolates with MIC (mg/L)									
Biocide	**Site**	0.125	0.25	0.5	1	2	4	8	16	32	>64
TRI	**1**										10
	2										9
	3										3
	4										2
BNZ	**1**								4	5	1
	2							1	4	1	1
	3									1	
	4								2		
$AgNO_3$	**1**						2	4	2		
	2					2	1	4	2		
	3				1	0	0	0	0	1	
	4				1	0	0	0	1		

The minimum inhibitory concentrations were determined for C. freundii isolates isolated from wastewater from four different sites at one hospital: Site 1: Radiology/Oncology; Site 2: Residential Site 3: Theatre/Emergency/Outpatient; and Site 4: Reference site. Vertical black lines indicate EUCAST ECOFF values [118] and red lines indicate ECOFF values for biocides as determined in this study. ECOFF value for triclosan could not be established due to intrinsic resistance. Four different antimicrobials were tested: chlorhexidine (CHL), triclosan (TRI), benzalkonium chloride (BNZ) and silver nitrate ($AgNO_3$) were used.

3.3.2 E. cloacae biocide MIC displays unimodal population

A total of 25 *E. cloacae* isolates were tested for resistance against 8 antibiotics across 5 antibiotic classes: cephalosporins, quinolones, carbapenems, aminoglycosides and polymyxin [Table 8]. A high level of resistance against cephalosporins, quinolones and aminoglycosides were seen, however, isolates from the reference site were not MDR. Against cephalosporins, some isolates were over 1000 times more resistant and against fluoroquinolones, some isolates were over 120 times more resistant than the EUCAST cut off value. At least 50% of isolates were resistant against both tested aminoglycosides and 48% of *E. cloacae* were up to 250 times more resistant against a carbapenem. In total the MDR isolates represented 73% (n=19) of the tested *E. cloacae* population [Appendix 3].

Table 8. Antibiotic susceptibility profile of 25 E. cloacae isolated from wastewater

		Number of *E. cloacae* isolates with MIC (mg/L)									
Antimicrobial agent	**Site**	0.125	0.25	0.5	1	2	4	8	16	32	>64
FEP	**1**					1					
	2	2	0	0	1	0	0	3	2	0	2
	3		1	1	1	1	0	1	0	0	5
	4	3	1								
CAZ	**1**										1
	2				1	1	1	0	0	0	7
	3						4	0	0	0	6
	4	1	2	0	0	0	0	1			
CIP	**1**			1							
	2	1	1	0	1	4	2	0	1		
	3	1	3	0	2	2	2				
	4	4	1								
LEV	**1**				1						
	2		1	2	1	0	5	1			
	3	1	1	3	2	1	0	1			
	4	5									
GEN	**1**										1
	2		1	0	0	0	0	0	0	2	7
	3		3	0	0	0	0	1	1	1	1
	4		2	2							
TOB	**1**									1	
	2				1	0	0	1	3	1	
	3		4	0	0	0	1	1	2	1	
	4		2	1	0	0	0	0	0	0	1
MER	**1**			1							
	2	4	1	0	1	2	1	0	1		
	3		3	0	0	0	3	0	0	1	
	4	2	1	0	1						
COL	**1**	1									
	2	7	0	0	1						
	3	5	0	1	0	1					
	4	2									

The minimum inhibitory concentrations were determined for E. cloacae isolates isolated from wastewater from four different sites at one hospital: Site 1: Radiology/Oncology; Site 2: Residential Site 3: Theatre/Emergency/Outpatient; and Site 4: Reference site. Vertical black lines indicate EUCAST ECOFF values [118]. Eight different antibiotics: cefepime (FEP), ceftazidime (CAZ), Ciprofloxacin (CIP), Levofloxacin (LEV), Gentamicin (GEN), Tobramycin (TOB), Meropenem (MER), Colistin (COL).

In the current study 25 MICs were performed with *E. cloacae* against four biocides: chlorhexidine, triclosan, benzalkonium chloride and silver nitrate [Table 9]. ECOFF values

were determined for biocides using the ECOFFFinder: 95% ECOFF for *E. cloacae* was 128mg/L against chlorhexidine, 1mg/L against triclosan and 32mg/L against $AgNO_3$. The ECOFF values for benzalkonium chloride were unable to be determined as the tested population all had MICs of 16mg/L [Figure 21]. The ECOFF values suggested unimodal population of sensitivity against chlorhexidine, triclosan and $AgNO_3$.

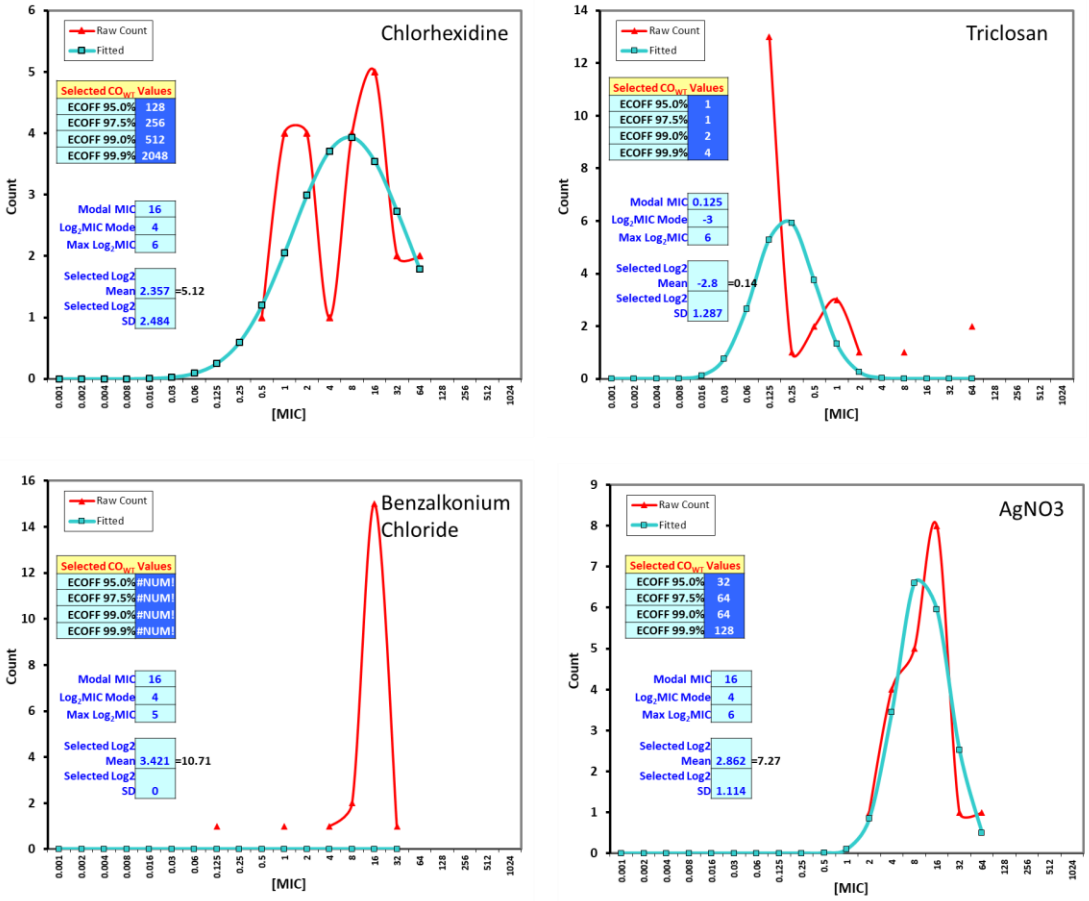

Figure 21. ECOFF values for *E. cloacae* against common biocides
The ECOFF values for chlorhexidine, triclosan, benzalkonium and $AgNO_3$ were found (with 95% cut-off) values, using the ECOFFFinderXL2010.xlsm tool (https://clsi.org/meetings/microbiology/ecoffinder/). The 95% ECOFF for E. cloacae was 128mg/L against chlorhexidine, 1mg/L against triclosan and 32mg/L against $AgNO_3$. The ECOFF values for benzalkonium chloride were unable to be determined as the tested population all had minimum inhibitory concentrations of 16mg/L.

Table 9. Biocide susceptibility profile of 25 E. cloacae isolated from wastewater

		Number of *E. cloacae* isolates with MIC (mg/L)									
Biocide	**Site**	0.125	0.25	0.5	1	2	4	8	16	32	>64
COL	**1**	1									
	2	7	0	0	1						
	3	5	0	1	0	1					
	4	2									
CHL	**1**								1		
	2				1	3	0	1	1	2	1
	3			1	2	0	0	2	1	0	1
	4				1	0	1	1	1		
TRI	**1**	1									
	2	5	0	1	2	0	0	1	0	0	1
	3	4	2	0	0	1					
	4	3	0	0	1						
BNZ	**1**									1	
	2							2	6		
	3				1	0	0	0	8		
	4	1	0	0	0	0	1	0	1		
$AgNO_3$	**1**								1		
	2							4	3	1	
	3					1	3	1	2	0	2
	4						1	0	2		

The minimum inhibitory concentrations were determined for E. cloacae isolates isolated from wastewater from four different sites at one hospital: Site 1: Radiology/Oncology; Site 2: Residential Site 3: Theatre/Emergency/Outpatient; and Site 4: Reference site. Vertical red lines indicate ECOFF values for biocides as determined in this study [Figure 22]. Four different biocides were tested: chlorhexidine (CHL), triclosan (TRI), benzalkonium chloride (BNZ) and silver nitrate ($AgNO_3$) were used.

3.3.3 E. coli from the reference site were sensitive and biocide MIC smaller in comparison to MIC of literature

A total of 36 *E. coli* were analysed for resistance against 9 antibiotics across 5 antibiotic classes: cephalosporins, quinolones, carbapenems, aminoglycosides and polymyxin [**Appendix 4**]. A total of 22% (n=8) were sensitive to all tested antibiotics, 44.4% (n=16) were MDR. All isolates were sensitive to colistin [Table 10].

Table 10. Antimicrobial susceptibility profile of 36 E. coli isolated from wastewater

		Number of *E. coli* isolates with MIC (mg/L)									
Antibiotic	**Site**	0.125	0.25	0.5	1	2	4	8	16	32	>64
FEP	**1**						1	0	0	0	2
	2	3	0	1	0	0	1	0	1	0	1
	3	9	0	0	2	3	0	0	0	1	1
	4	3	3								
CAZ	**1**									1	2
	2	2	1	0	0	0	0	0	0	1	4
	3	2	7	3	0	0	0	0	0	2	5
	4	1	4	0	0	0	0	0	1		
CIP	**1**			1	1	0	0	0	1		
	2	4	1	0	2	0	0	0	0	0	1
	3	4	2	6	2	1	2	1	1		
	4	5	0	0	0	0	0	0	1		
LEV	**1**				1	1					
	2	5	1	0	1	0	0	0	1		
	3	5	7	2	2	1	0	0	1		
	4	4	1	0	0	0	0	0	1		
GEN	**1**								1	0	2
	2	1	1	1	1	0	0	0	0	0	4
	3	1	1	2	4	1	2	0	1	0	7
	4	1	0	1	3	0	1				
TOB	**1**					0	0	0	1	2	
	2			2	1	0	0	0	2	0	1
	3			2	6	3	0	1	4	2	
	4	0	2	0	3	2					
MER	**1**	1	1								
	2	8									
	3	8	4	3	1	1	0	0	0	0	1
	4	6									
COL	**1**	1	2								
	2	7	1								
	3	15	1								
	4	6									

The minimum inhibitory concentrations were determined for E. coli isolates isolated from wastewater from four different sites at one hospital: Site 1: Radiology/Oncology; Site 2: Residential Site 3: Theatre/Emergency/Outpatient; and Site 4: Reference site. Vertical black lines indicate EUCAST ECOFF values [118]. Eight different antibiotics: cefepime (FEP), ceftazidime (CAZ), Ciprofloxacin (CIP), Levofloxacin (LEV), Gentamicin (GEN), Tobramycin (TOB), Meropenem (MER), Colistin (COL).

In the current study 36 MICs were performed with *E. coli* against four different biocides [Table 11]. According to the study by Morrissey [91], the ECOFF of *E. coli* is 64mg/L against chlorhexidine, 2mg/L against triclosan and 64mg/L against benzalkonium chloride. From this study, the ECOFFFinder determined the 95% *ECOFF* of *E. coli* to be 1mg/L

against chlorhexidine, 0.25mg/L against triclosan 32mg/L against benzalkonium chloride and 128mg/L against $AgNO_3$ [Figure 22]. It is interesting to observe this study found the MIC of *E. coli* against chlorhexidine to be significantly smaller than found by Morrissey. This may be attributed to a smaller sample population but differences may be attributed be because of sampling from a different population, location and conditions [91]. The mentioned study found the MIC to be 64mg/L whereas in the current study, 76.4% of isolates had MIC of ≤ 1mg/L against chlorhexidine, isolates displayed MICs as low as 0.25mg/L, 256 times lower than the MIC from Morrissey [91]. Comparing the percentages of isolates from this study with MIC less than or equal to the MIC of literature, majority of isolates from this study had smaller MICs that is: for triclosan, 97% of isolates (n=33), for chlorhexidine, 94.3% (n=33) and for benzalkonium chloride, there were 87.5% (n=28) of isolates.

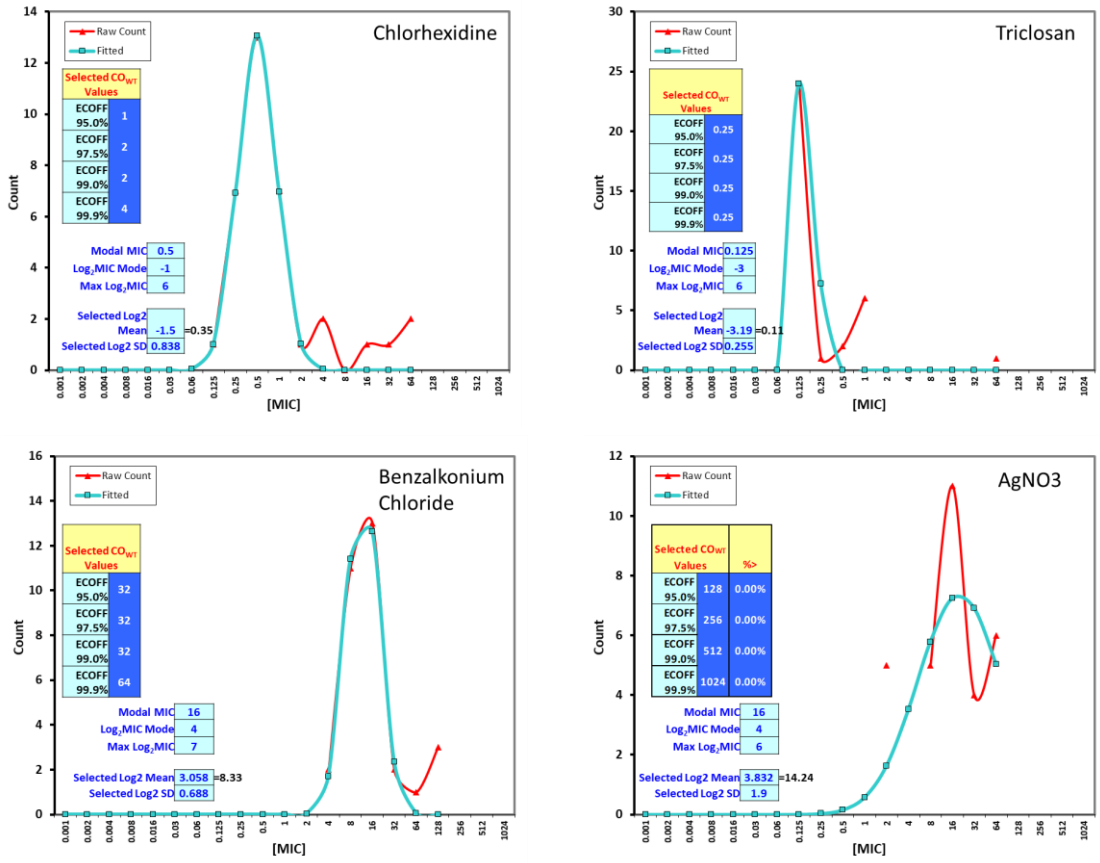

Figure 22. ECOFF values for E. coli against common biocides
The ECOFF values for chlorhexidine, triclosan, benzalkonium chloride and $AgNO_3$ were found (with 95% cut-off) values, using the ECOFFFinderXL2010.xlsm tool (https://clsi.org/meetings/microbiology/ecoffinder/). The 95% ECOFF for E. coli was 1mg/L against chlorhexidine, 0.25mg/L against triclosan, 32mg/L against benzalkonium chloride and 128mg/L against $AgNO_3$.

Table 11. Biocide susceptibility profile of 36 E. coli isolated from wastewater

		Number of *E. coli* isolates with MIC (mg/L)									
Biocide	**Site**	0.125	0.25	0.5	1	2	4	8	16	32	>64
CHL	**1**			2	1						
	2		1	0	3	1	1				
	3		5	10	3	0	1				
	4		2	5							
TRI	**1**	2									
	2	3	0	0	4						
	3	16	0	1	2						
	4	5	0	0	1						
BNZ	**1**							1	1	0	1
	2							2	1	1	
	3						2	8	9		
	4										2
$AgNO_3$	**1**							1	1	0	1
	2							2	3	1	
	3					3	0	1	11	3	1
	4										4

The minimum inhibitory concentrations were determined for E. coli isolates isolated from wastewater from four different sites at one hospital: Site 1: Radiology/Oncology; Site 2: Residential Site 3: Theatre/Emergency/Outpatient; and Site 4: Reference site. Vertical red lines indicate ECOFF values for biocides as determined in this study [Figure 22]. Four different biocides were tested: chlorhexidine (CHL), triclosan (TRI), benzalkonium chloride (BNZ) and silver nitrate ($AgNO_3$) were used.

3.3.4 K. pneumoniae highly resistant against antimicrobials and triclosan

A total of 34 *K. pneumoniae* isolates were tested for resistance against 9 antibiotics across 5 antibiotic classes: cephalosporins, quinolones, carbapenems, aminoglycosides and polymyxin [Table 12]. All isolates from Site 1 were up to 1000 times more resistant than EUCAST breakpoint values against cephalosporins, fluoroquinolones and aminoglycosides. 77% of isolates were resistant against 1 or more classes of antibiotics. 59% (n=20) of isolates were resistant to three or more classes of antibiotics and 26% (n=9) were resistant to 4 or more. Approximately 21% (n=7) of isolates were completely sensitive to all tested antibiotics [**Appendix 5**].

Table 12. Antibiotic susceptibility profile of 34 K. pneumoniae isolated from wastewater

		Number of *K. pneumoniae* isolates with MIC (mg/L)									
Antibiotic	**Site**	0.125	0.25	0.5	1	2	4	8	16	32	>64
FEP	**1**		0	0	0	0	0	0	1	1	4
	2	1	3	1	2	3	1	1	3	2	1
	3	2	0	1	0	1	1	0	0	0	2
	4	3									
CAZ	**1**								2	0	4
	2		1	2		2	1	1	1	0	10
	3			2	0	0	0	0	0	0	5
	4	2	1								
CIP	**1**					1	1	1	0	1	2
	2	7	2	3	2	2	0	8			
	3	.	.	.	.	.	.	.	.	.	.
	4	.	.	.	.	.	.	.	.	.	.
LEV	**1**			1	0	1	1	1	0	2	
	2	6	3	2	1	5	1				
	3	3	0	2	2						
	4	3									
GEN	**1**									2	4
	2	1	0	1	3	0	0	1	1	1	10
	3		1	1	0	0	0	1	1	0	3
	4	2	1								
TOB	**1**						1	0	1	0	4
	2	2	0	0	1	2	4	2	2	3	1
	3	1	1	0	0	0	0	0	2	1	2
	4	2	0	1							
MER	**1**	2	3	1							
	2	11	5	2							
	3			4							
	4	3									
COL	**1**	1	3	1	1						
	2	8	8	1	1						
	3	5	2								
	4	2	1								

The minimum inhibitory concentrations were determined for 34 K. pneumoniae isolates isolated from wastewater from four different sites at one hospital: Site 1: Radiology/Oncology; Site 2: Residential Site 3: Theatre/Emergency/Outpatient; and Site 4: Reference site. Vertical black lines indicate EUCAST ECOFF values[118]. Eight different antibiotics: cefepime (FEP), ceftazidime (CAZ), ciprofloxacin (CIP), levofloxacin (LEV), gentamicin (GEN), tobramycin (TOB), meropenem (MER), colistin (COL).

Sensitivity to biocides were tested; 34 were tested against triclosan, 27 were tested against benzalkonium chloride [Table 13]. According to the study by Morrissey [91], the ECOFF

of *K. pneumoniae* is 64mg/L against chlorhexidine, 2mg/L against triclosan and 32mg/L against benzalkonium chloride [91]. From this study, the ECOFFFinder determined the 95% *ECOFF* of *K. pneumoniae* to be 64mg/L against chlorhexidine, 16mg/L against triclosan, against benzalkonium chloride and 16mg/L against $AgNO_3$ [Figure 23]. In comparison to Morrissey, the ECOFF value of this population of *K. pneumoniae* against triclosan was eight-fold bigger compared to Morrissey. This is likely explained by isolation of *K. pneumoniae* wild types from a highly selective environment.

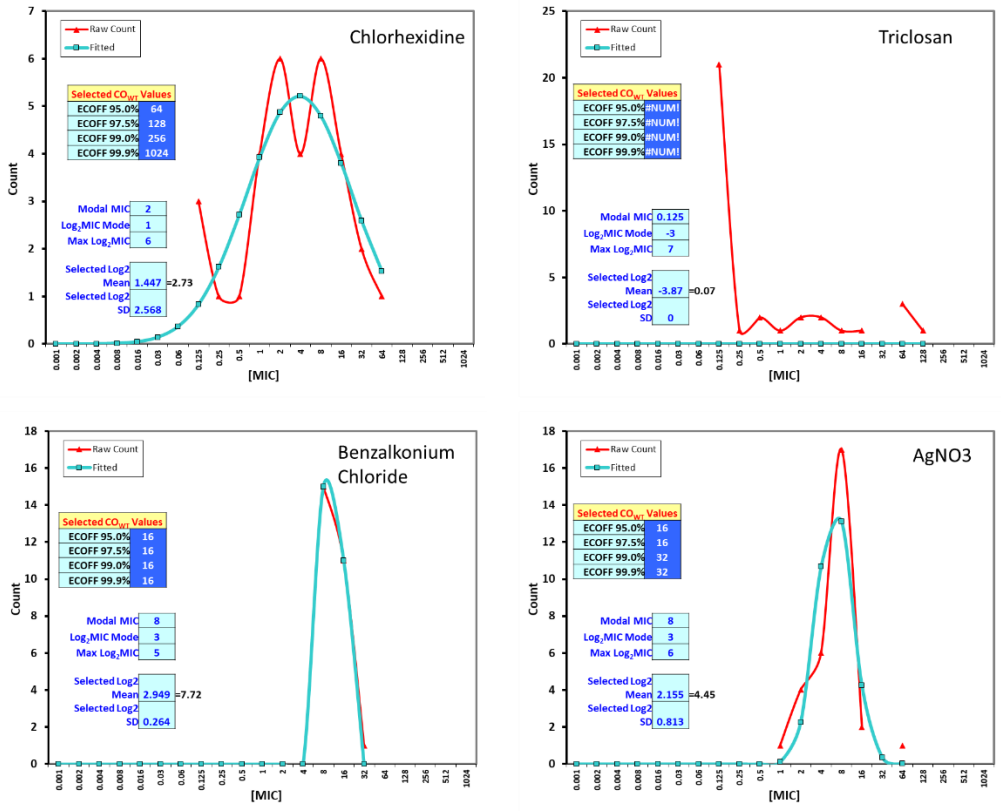

Figure 23. ECOFF values for K. pneumoniae against common biocides
The ECOFF values for chlorhexidine, triclosan, benzalkonium and $AgNO_3$ were found (with 95% cut-off) values, using the ECOFFFinderXL2010.xlsm tool (https://clsi.org/meetings/microbiology/ecoffinder/). The 95% ECOFF for K. pneumoniae was 64mg/L against chlorhexidine, 16mg/L against benzalkonium chloride and 16mg/L against $AgNO_3$. The ECOFF value for triclosan was not able to be determined by ECOFFFinder.

Table 13. Biocide susceptibility profile of 34 K. pneumoniae isolated from wastewater

		Number of *K. pneumoniae* isolates with MIC (mg/L)									
Biocide	**Site**	0.125	0.25	0.5	1	2	4	8	16	32	>64
CHL	**1**				1		2	1	2		
	2	1	1	1	2	2	2	4	2	2	
	3	2	0	0	1	4	0	1			
	4										1
TRI	**1**	3	0	1	0	1	0	0	0	0	1
	2	7	2	1	1	1	1	1	1	0	2
	3	5	1	0	0	0	1				
	4	3									
BNZ	**1**							3	2		
	2							7	7	1	
	3							4	2	.	
	4					0	0	1			
$AgNO_3$	**1**				1	1	0	3			
	2					3	2	8	2	0	1
	3						3	4			
	4						1	2			

The minimum inhibitory concentrations were determined for K. pneumoniae isolates isolated from wastewater from four different sites at one hospital: Site 1: Radiology/Oncology; Site 2: Residential Site 3: Theatre/Emergency/Outpatient; and Site 4: Reference site. Vertical red lines indicate ECOFF values for biocides as determined in this study [Figure 23]. ECOFF value for triclosan could not be established. Four biocides were tested: chlorhexidine (CHL), triclosan (TRI), benzalkonium chloride (BNZ) and silver nitrate ($AgNO_3$) were used.

3.3.5 K. oxytoca MIC profiling revealed multidrug and biocide resistant isolates

A total of 88 *K. oxytoca* isolates were tested for resistance against 9 antibiotics across 5 antibiotic classes: cephalosporins, quinolones, carbapenems, aminoglycosides and polymyxin. 77% of isolates were resistant against 1 or more classes of antibiotics. 67% (n=59) of isolates were resistant to three or more classes of antibiotics and 24% (n=22) were resistant to 4 or more. Approximately 23% (n=20) of isolates were completely sensitive to all tested antibiotics. However, the rates of resistance were not evenly distributed amongst the different sites: 82% of isolates from Site 2 were completely sensitive to all 5 classes of antibiotics; 56% of isolates from Reference site were completely sensitive to all 5 classes of antibiotics; 97% of isolates from Site 1 were resistant to 3 or more classes of antibiotics; 84% of isolates from Site 3 were resistant to 3 or more classes of antibiotics. In total, 67% (n=59) isolates were MDR.

Table 14. Antibiotic susceptibility profile 88 of K. oxytoca isolated from wastewater

		Number of *K. oxytoca* isolates with MIC (mg/L)									
Antibiotic	**Site**	0.125	0.25	0.5	1	2	4	8	16	32	>64
FEP	**1**		0	0	0	0	0	6	3	1	26
	2	11	0	1	1	0					
	3	1	1	3	0	3	2	2	3	3	7
	4	13	0	2	0	1					
CAZ	**1**							1	1	1	34
	2	6	3	0	0	0	0	0	0	0	2
	3	1	1	1	0	1	1	2	1	2	16
	4	8	5	1	0	0	0	0	2		
CIP	**1**		0	2	0	6	11	7	2	3	5
	2	11	0	2							
	3	2	2	2	0	2	2	3	6	4	2
	4	12	1	2							
LEV	**1**	1	1	4	14	10	1	2	1	2	
	2	10	1	1	0	1					
	3	4	1	0	5	4	3	5	3		
	4	12	0	2	0	1					
GEN	**1**					1	0	1	0	4	30
	2		3	3	1	1	0	0	0	0	2
	3	1	2	2	0	0	0	0	2	2	16
	4	2	2	4	8						
TOB	**1**						1	3	10	12	10
	2	2	1	3	3			2			
	3		2	2		1	3	2	4	8	3
	4	7	1	6	2						
MER	**1**	17	4	6	4	5					
	2	10	1								
	3	11	4	5	3	1	1				
	4	16									
IMI	**1**	14	14	8							
	2	2	4	4	1						
	3		4	18	2	1					
	4		8	8							
COL	**1**	31	5								
	2	9	2								
	3	25									
	4	10	5	1							

The minimum inhibitory concentrations were determined for K. oxytoca isolates isolated from wastewater from four different sites at one hospital: Site 1: Radiology/Oncology; Site 2: Residential Site 3: Theatre/Emergency/Outpatient; and Site 4: Reference site. Vertical black lines indicate EUCAST ECOFF values[118]. Nine different antibiotics: cefepime (FEP), ceftazidime (CAZ), ciprofloxacin (CIP), levofloxacin (LEV), gentamicin (GEN), tobramycin (TOB), meropenem (MER), imipenem (IMI) and colistin (COL).

To our knowledge, no official ECOFF value is available for *K. oxytoca* against chlorhexidine, triclosan, benzalkonium chloride or $AgNO_3$. One paper has found the MIC to be around 30mg/L [77]. In this current study 90 MICs were performed with *K. oxytoca* against chlorhexidine [Table 13]. The calculated 95% ECOFF using the ECOFFFinderXL2010.xlsm, Figure 24, showed an MIC of 32 mg/L, a similar result to the previously quoted study. In this study, 88 isolates were tested against triclosan and the ECOFF for 95% of isolates was found to be at 0.5 mg/L.

To our knowledge, no ECOFF value is available for *K. oxytoca* against benzalkonium chloride. In this study, 81 isolates were tested against benzalkonium chloride and the ECOFF for 95% of isolates was found to be at 32 mg/L. The ECOFFFinder indicated the tested population had unimodal resistance against benzalkonium chloride. To our knowledge, no ECOFF value is available for *K. oxytoca* against $AgNO_3$. In this study, 85 isolates were tested against $AgNO_3$ and the ECOFF for 95% of isolates was found to be at 32 mg/L [Figure 24].

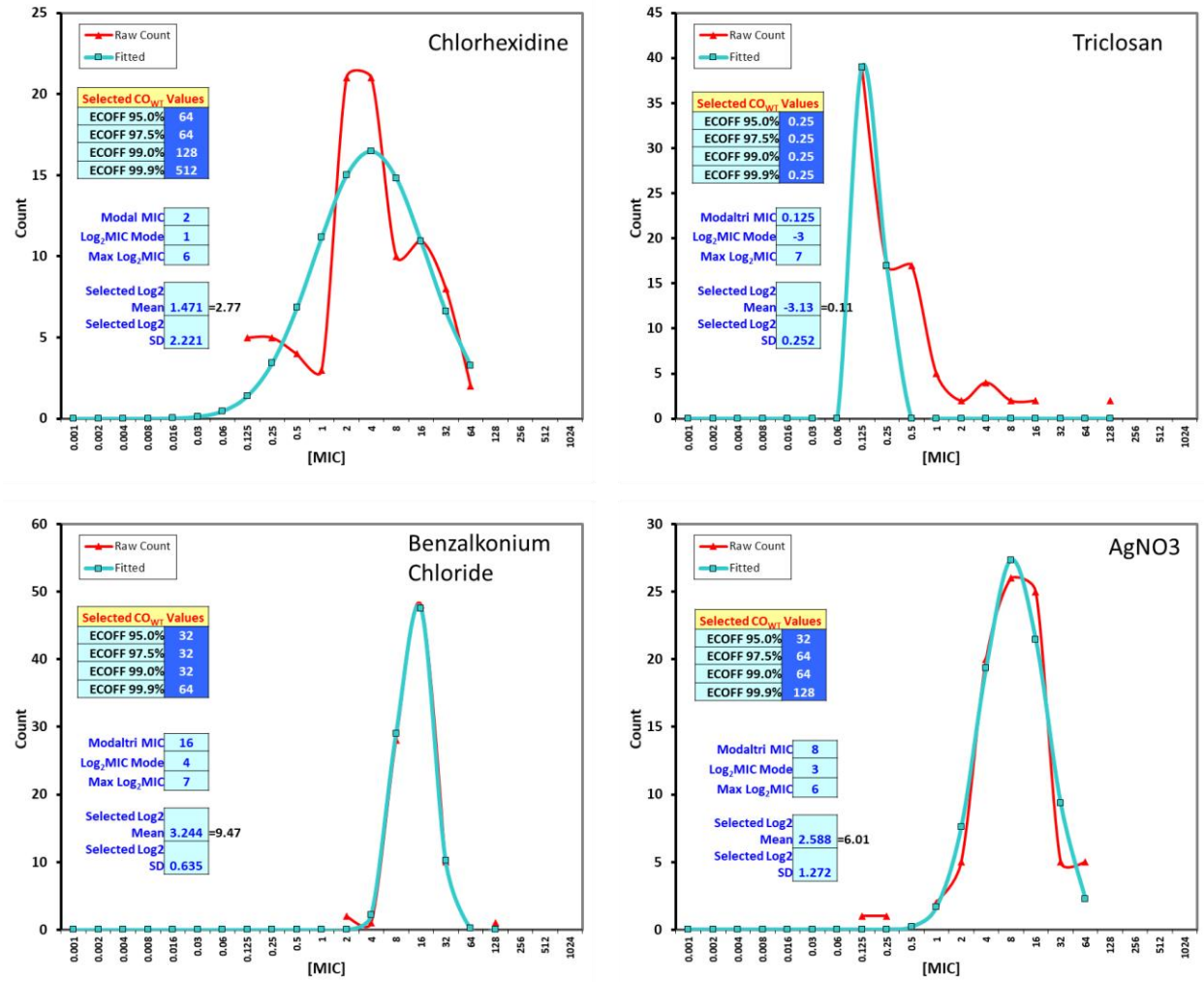

Figure 24. **ECOFF values for *K. oxytoca* against common biocides**

The ECOFF values for chlorhexidine, triclosan, benzalkonium and $AgNO_3$ were found (with 95% cut-off) values, using the ECOFFFinderXL2010.xlsm tool (https://clsi.org/meetings/microbiology/ecoffinder/). The 95% ECOFF for *K. oxytoca* was 64mg/L against chlorhexidine, 0.25mg/L against triclosan, 32mg/L against benzalkonium chloride and 32mg/L against $AgNO_3$.

Table 15. Biocide susceptibility profile of 88 K. oxytoca isolated from wastewater

		Number of *K. oxytoca* isolates with MIC (mg/L)									
Antimicrobial agent	**Site**	0.125	0.25	0.5	1	2	4	8	16	32	>64
CHL	**1**	1	3	2	0	5	7	5	6	5	2
	2		1		1	2	4	1	1		
	3		2	1	2	8	5	2	4	3	
	4	3	0	0	1	6	4	2			
TRI	**1**	20	6	5		1	2	1	1		
	2	8	2	0	0	0	0	0	0	0	1
	3		5	12	4	1	2	1	1	0	1
	4	10	5	0	1						
BNZ	**1**							4	20	9	1
	2							7	4		
	3					2	1	10	14		
	4								5	10	1
$AgNO_3$	**1**				1	3	11	9	8	2	2
	2					1	0	3	6		1
	3	1	1	0	1		6	9	6	1	2
	4					1	3	5	5	2	

The minimum inhibitory concentrations were determined for 88 K. oxytoca isolates isolated from wastewater from four different sites at one hospital: Site 1: Radiology/Oncology; Site 2: Residential Site 3: Theatre/Emergency/Outpatient; and Site 4: Reference site. Vertical red lines indicate ECOFF values for biocides as determined in this study [Figure 24]. Four biocides were tested: chlorhexidine (CHL), triclosan (TRI), benzalkonium chloride (BNZ) and silver nitrate ($AgNO_3$) were used.

3.4 Analysis of antibiotic resistance amongst *K. oxytoca*

Due to the prevalence of *K. oxytoca* as an emerging pathogen and the high level of resistance from the MIC results [Table 14], this study focused further on *K. oxytoca* to understand its potential for drug resistance and investigate its ability to carry resistance genes (Chapter 4). As of late 2022, *K. oxytoca* was identified as a complex containing at least nine species of *Klebsiella* [126, 128]. Current clinical and genomic methods are unable to clearly distinguish species within this complex due to their similarity in phenotypic and genomic characteristics; the complex will continue to be referred to here as *K. oxytoca*. The MICs of *K. oxytoca* revealed distinct populations between different hospital sites; a significantly higher proportion of resistant populations were present within Site 1 and Site 3 compared to Site 2 and 4 for the tested antibiotics cefepime, ceftazidime, ciprofloxacin, levofloxacin, gentamicin, tobramycin, meropenem and imipenem [Figure 25]. Furthermore, 100% (n=36) and 92% (n=22) of isolates from Site 1 and Site 3 respectively were resistant to 2 or more classes of antibiotics [Figure 26]. This pattern was distinct from Site 2 and Site 4 where only 18% and 6% (n=1) respectively, were resistant against 2 or more classes of antibiotics. Both sites had a large proportion of isolates completely sensitive to all 9 antibiotics tested; site 2 had 82% (n=9) and site 4 had 69% (n=11) of isolates without any phenotypic antibiotic resistance. This is contrasts Site 1 where no organisms were completely sensitive to a class of antibiotic and Site 3 where only 8% were sensitive to all antibiotic classes. The higher proportion of resistant isolates from two different hospital sites Radiology/Oncology and Emergency/Theatre is representative of the environmental niches in which they originate from. Figure 26 further highlights the propensity of an emerging pathogen, such as *K. oxytoca*, to be subject to selective pressures and development of resistance mechanisms. Overall, this illustrates and supports the notion and significance of environmental selective pressures on the development and/or the dissemination of antibiotic resistance genes amongst bacterial organisms.

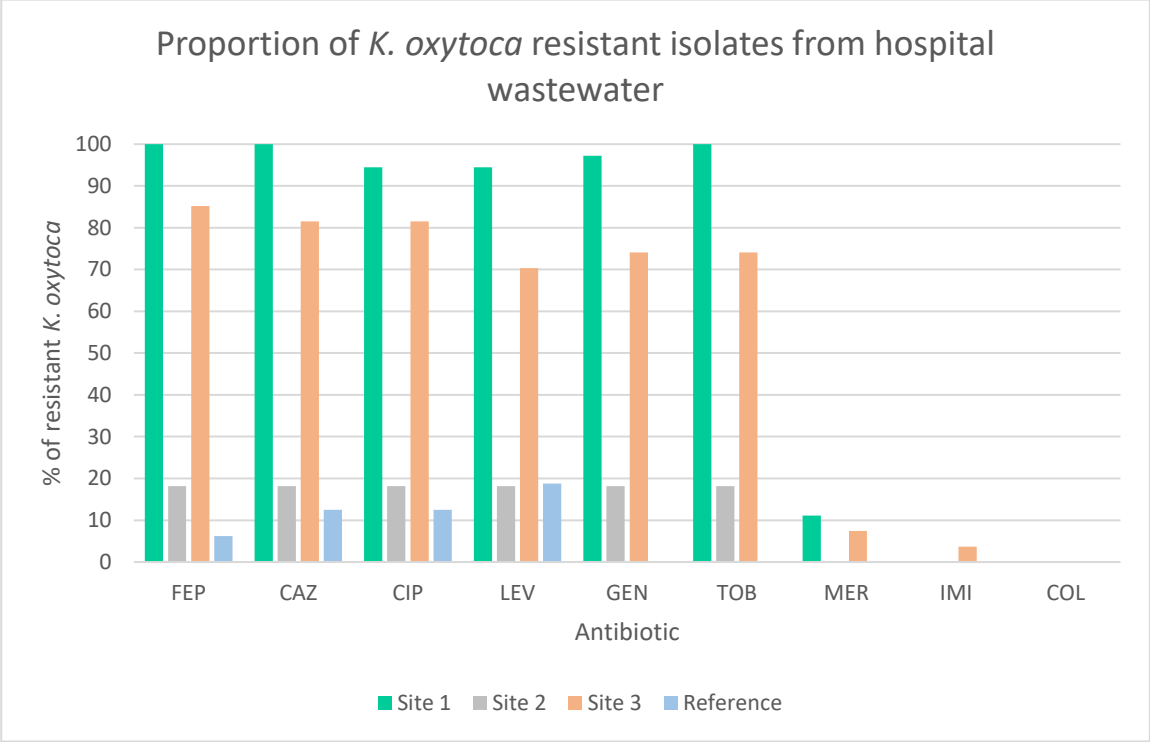

Figure 25. Proportion of resistance against 9 antibiotics of K. oxytoca isolated from different wastewater sites from a major metropolitan hospital

Results from minimum inhibitory concentrations of K. oxytoca (n=88) revealed the proportions of resistance varied dependent on the environment they were isolated from. Site 1: Radiology/Oncology and Site 3: Theatre/Outpatient/Emergency had higher proportions of resistance compared to isolates from Site 2: Residential, and Site 4 Reference site. The susceptibility against the K. oxytoca isolates were based on the 2020, European Committee on Antimicrobial Susceptibility Testing breakpoint values. FEP: Cefepime; CAZ: Ceftazidime; CIP: Ciprofloxacin; LEV: Levofloxacin; GEN: Gentamicin; TOB: Tobramycin; MER: Meropenem; IMI: Imipenem; COL: Colistin.

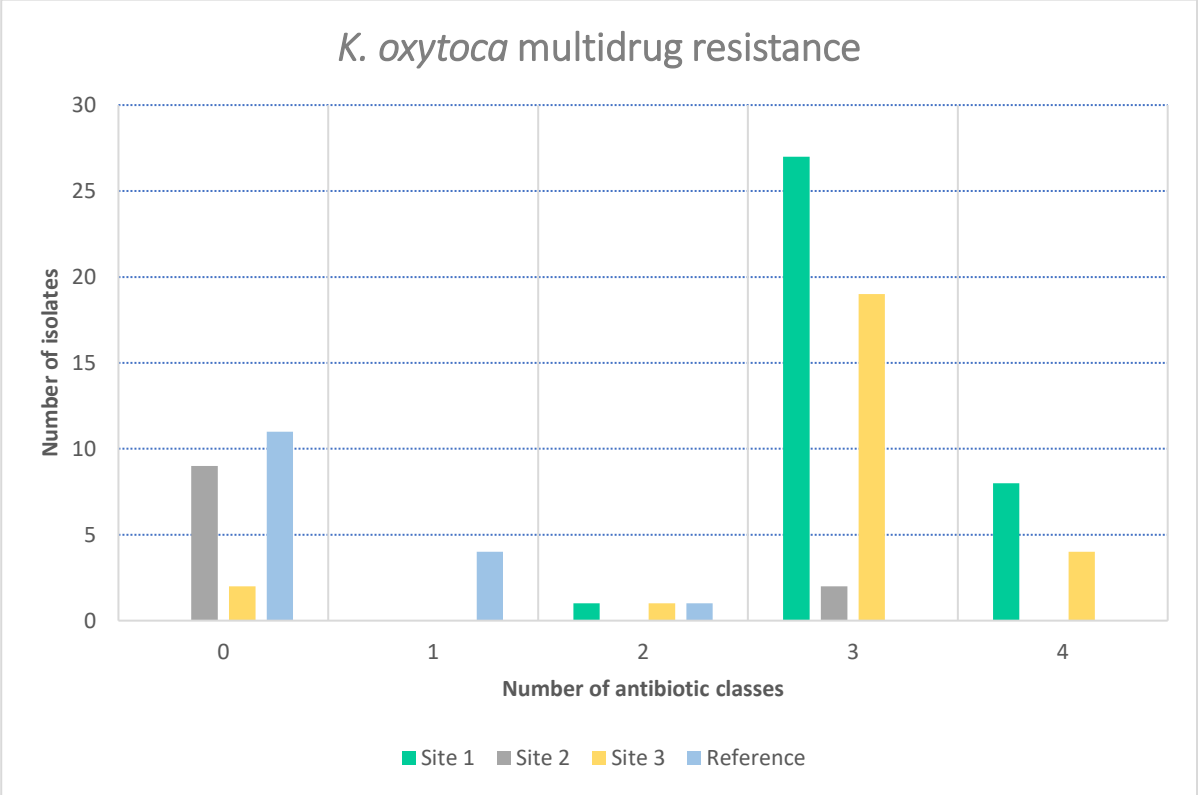

Figure 26. Number of K. oxytoca isolates resistant against multiple antibiotic classes

The number of isolates resistant against numbers of antibiotic classes were different from wastewater from different hospital sites. These results were based off from minimum inhibitory concentrations of K. oxytoca (n=88). Site 1: Radiology/Oncology and Site 3: Theatre/Outpatient/Emergency had higher proportions of multidrug resistance compared to isolates from Site 2: Residential, and Site 4 Reference site.

3.5 Summary of isolation and phenotypic resistance results

Chapter 3 explored the importance of culture-based methods for phenotypic surveillance of MDR organisms through use of selective and differential media. Tergitol-7 was a highly useful medium for isolating a variety of organisms from wastewater and was able to capture organisms with a wider range of pathogenic and environmental isolates. The use of selective and differential media for isolating organisms of interest successfully recovered 1065 organisms from a total of 102 different species. The most prevalent being *Pseudomonas spp*. (n=216), *Klebsiella spp*. (n=170) and *Acinetobacter spp*. (n=111). Emerging opportunistic pathogens and environmental species such as *P. putida* (n=43) and *K. oxytoca* (n=124) were recovered in higher proportions compared to organisms traditionally considered a large problem in healthcare such as *Acinetobacter spp.* [149]. Different proportions of bacterial genera were recovered from each site with culture-based methods. *Pseudomonas spp*. were the most frequently isolated species, 30 different *Pseudomonas* species represented 20.3% of all isolates recovered. The most common *Pseudomonas spp*.

were *P. aeruginosa* and *P. putida*. Of the other notable species recovered: 16% were *Klebsiella spp.*: *K. oxytoca, K. pneumoniae* and *K. variicola*; 10.4% *Acinetobacter spp.: A. towneri, A. johnsonii* and *A. junii*; 8.3% were *Citrobacter spp.: C. freundii, C. braakii, C. amalonaticus* and *C. farmeri, C. gillenii;* 5.9% *Enterococcus spp.: E. faecium, E. casselifalus;* 4.8% were *Escherichia spp., E. coli, E. hermannii* and *E. vulneris*; 3.6% were *Enterobacter spp.: E. asburiae, E. cloacae* and *E. kobei.* Further analysis was performed with metagenomic data. Through culture-based methods and metagenomic data, this study showed a distinct difference in resistance profiles between different wastewater sampling sites. Species diversity between different wastewater environments was supported by 16s profiling and the phenotypic data. 16s profiling was able to identify phylum and some bacterial populations were able to be discerned as far as the genus level (Bacterial taxonomy is ranked as follows: Domain, Phylum, Class, Order, Family, Genus and Species). The use of 16s along with the phenotypic data was able to illustrate a few things; firstly, it demonstrated the true complexities of the wastewater environment; secondly, it illustrated there was indeed a major difference between each site in regard to frequency of bacterial populations and finally, that the laboratory-based methodology used in this study was an effective way to select for organisms of interest and to minimise the isolation of other organisms. Based on phylum data from 16s profiling, the sites varied as follows: *Firmicute* frequency: S1-3.11%, S2-40.84%, S3-59.02%, R-66.87%; *Proteobacteria* frequency: S1-54.23%, S2-42.74%, S3-25.08% and R -26.51%. According to the 16s results, the reference site contained the highest frequency from the *Firmicute* phyla (*Lactobacillus'*s and *Streptococcus*); S1-1.72%, S2- 4.47%, S3-4.9% and R- 33.42%. These results are consistent with what is expected as normal flora within the human gut is 90% composed of bacteria from the *Firmicutes* and *Bacteroidetes* phyla [189]. The major phyla, *Firmicute, Proteobacteria, Bacteriodetes* and *Actinobactera,* identified across the 4 sites in this study were similar to those identified in other hospital wastewater but differed int their relative abundance [147].

The *Klebsiella oxytoca* complex is a complex consisting of nine species of *Klebsiella* with the *Klebsiella oxytoca* species the most prevalent of the nine [126]. As demonstrated in Chapter 3.2.2, the *Klebsiella* genus represented 16% of isolated organisms, the largest genus found within the hospital wastewater environment. Of that number, *K. oxytoca* was the most prevalent species identified, as according to identification by the MALDI-TOF, representing 72.9% of the total isolated *Klebsiella*. *K. oxytoca* is an emerging, opportunistic pathogen, responsible for hospital acquired infections in vulnerable populations [131, 132]. In this

study, most *K. oxytoca* isolates showed resistance to at least one antibiotic (75% n=67) and a very high proportion of (95% n=63) of isolates were also MDR. Furthermore, the well established link between biocide resistance, cross resistance and MDR is supported within this study as 72% (n=64) of resistant isolates were simultaneously MDR as well [21, 74, 76]. Studies have demonstrated the potential for clinical organisms to acquire resistance determinants from environmental or emerging pathogens within selective environments [33] and a recent study from this group demonstrated cross-resistance between antibiotics and biocides from Enterobacterales, *P. aeruginosa*, from different ecological niches [17]. Thus, the prevalence of *K. oxytoca* in this study, where organisms of clinical origin are abundant, is severely concerning. To investigate the presence of AMR genes, sequence analysis was performed on *K. oxytoca* isolates in Chapter 4.

To characterise antimicrobial phenotypes, MIC broth dilutions were performed to investigate phenotypic resistance patterns, assess resistance levels against common antibiotics and ascertain the presence of multidrug resistance within different populations from different wastewater sites. The phenotypic resistance profiles of *Enterobacterales - C. freundii, E. cloacae, E. coli, K. oxytoca and K. pneumoniae* were all analysed. A very high level of resistance was found in isolates, for instance, *K. pneumoniae* and *E. cloacae* were over 1000 times more resistant against cephalosporins, than the higher range of the EUCAST ECOFF value. Multidrug resistance was prevalent amongst all species isolated from wastewater with 84% *C. freundii*, 73% *E. cloacae*, 44.4% of *E. coli*, 59% of K. pneumoniae and 67% of *K. oxytoca* isolates multidrug resistant. Furthermore, distinct differences in sensitive or resistant populations were observed at the different wastewater sites. This is seen distinctly in *K. oxytoca* where 56% and 82% of isolates from the Reference site and Site 2 were completely antibiotic sensitive, in contrast to 97% of isolates from Sites 1 and 3 being multidrug resistant. This is in line with studies which find ecological niches are influenced by the local selective pressures [190].

The determination of biocide ECOFFs was performed according to the methods outlined by Morrissey [91]. In this study, biocide ECOFF values were found for 'clinically relevant' isolates including *Salmonella spp., E. coli, K. pneumoniae* and *S. aureus*, thus this is the first time a study has explored the biocide ECOFF values for *K. oxytoca* isolates. As literature ECOFF values were unavailable, values obtained in this study were compared to the ECOFFs of *K. pneumoniae* by Morrissey. In comparison, the *K. oxytoca* isolates had generally slightly lower ECOFFs: of 32mg/L CHL (compared to 64mg/L for *K.*

pneumoniae), 0.5mg/L triclosan (compared to 2mg/L for *K. pneumoniae*), 32mg/L benzalkonium chloride (compared to 64mg/L for *K. pneumoniae*) and 32mg/L $AgNO_3$ (compared to 16mg/L by Morrissey).

Chapter 3 demonstrated a striking difference in proportions of multidrug resistant isolates amongst the different sampling sites and sites with the high multidrug resistance also had the highest diversity of organisms. Isolates from Site 2 and Reference were sensitive to most tested agents compared to those from Site 1 and 3 which displayed a very high level of resistance. This possibly could be due to the higher level of selective pressure in Site 1 and 3 and the fact that organisms such as *Klebsiella spp.* are capable of up taking many large plasmids [191]. Similarly, *E. coli*, *K. pneumoniae* and *E. cloacae* isolates recovered from the Reference site were not MDR and were all sensitive strains [Figure 25]. The Reference site, receiving no wastewater from the hospital site, would presumably contain fewer antibiotic metabolites, trace chemicals and other substances that go down hospital drains. Furthermore, hospital wastewater receives waste from patients with a higher likelihood of carrying multidrug resistant organisms than the general population [192], thus the reference site would be expected to be less selective environment compared to samples from hospitals. This preliminary finding supports what is recently accepted in literature, environments with high levels of selective pressures harbour growth conditions suitable for the selection of highly resistant and MDR bacterial populations [17, 110] and hospital wastewater contains higher resistance than municipal wastewater [149]. Future analysis of the chemical composition of the samples may help draw direct links regarding the effects that specific hospital wastewater environments have on the formation and dissemination of resistance genes.

Chapter 4: Genotypic analysis of *Klebsiella oxytoca*

4.0 Introduction

Results from Chapter 3 demonstrated the phenotypic resistance profiles of organisms isolated from hospital generated wastewater. To gain a greater understanding of resistance, it was of interest to characterise and explore the molecular mechanisms responsible for the phenotypic resistance. This project focused on the emerging, nosocomial pathogen *K. oxytoca*. As of late 2022, *K. oxytoca* was identified as a complex containing at least nine species of *Klebsiella* [126, 128]. However, current clinical and genomic methods are unable to clearly distinguish species within this complex due to their similarity in phenotypic and genomic characteristics. As the genotypic analysis in this study was also not able to discern *K. oxytoca* (as identified by MALDI-TOF), from other species within the complex, the complex will continue to be referred to here as *K. oxytoca*. *K. oxytoca* is an emerging pathogen with a large genome, typically 6 million base pairs [193], harbours multiple plasmids and has a large potential for the dissemination of MDR genes [126]. Relatively few studies have focused on *K. oxytoca* however as it was highly prevalent in our hospital samples and observed to be resistant against a number of antibiotics assessed in this study (Chapter 3), it was chosen for further analysis. *K. oxytoca* isolates (n=88) had distinct resistance populations amongst the different hospital wastewater sites. All (n=36) and 92% (n=22) of isolates from Site 1 and Site 3 respectively were resistant to 2 or more classes of antibiotics, this was distinct from Site 2 and Site 4 where a large proportion of isolates were completely sensitive to all 9 antibiotics tested; site 2 had 82% (n=9) and site 4 had 69% (n=11) of isolates without any phenotypic antibiotic resistance. Furthermore, a few isolates were resistant against biocides, at over 1000x the ECOFF values found in this study. Through further phenotypic and genotypic characterisation, this chapter aims to determine the genotypic resistance determinants of *K. oxytoca* isolates. Based on the data from Chapter 3, eight isolates were chosen based on their resistance profiles and their resistome determined using whole genome sequencing.

4.1 Results

4.1.1 Selection of isolates to be sequenced

Based on the data from Chapter 3, eight isolates were chosen based on their resistance profiles and their resistome determined using whole genome sequencing [Table 16].

KO356: Originally recovered from Site 1 from BileV+ agar and displayed extreme, phenotypic multidrug resistance, being highly resistant against cephalosporins, fluoroquinolones, aminoglycosides, carbapenems but sensitive to polymyxins. KO356 displayed high MICs against chlorhexidine and benzalkonium chloride compared to other *K. oxytoca* isolates.

KO459: From Site 2, recovered from BileV+ agar and was sensitive to every antibiotic tested. KO459 was of interest due to its high level of tolerance to Triclosan, displaying an MIC of 512mg/L.

KO480 and KO488: From Site 2, recovered from Terg0 agar and displayed resistance against cephalosporins, fluoroquinolones, aminoglycosides but was sensitive to carbapenems and polymyxins. This was of interest for two reasons: first, all tested *K. oxytoca* isolates from Site 2 were phenotypically sensitive to antibiotics besides KO480 and KO488. Secondly, KO480 had a very similar MIC profile to KO488 and its clonal linkage was of interest.

KO611: From Site 3, recovered from TergC+ agar and displayed extreme levels of drug resistance, being highly resistant against cephalosporins, fluoroquinolones, aminoglycosides, carbapenems but sensitive to polymyxins.

KO623: From Site 3, recovered from TergC+ agar and displayed phenotypic multidrug resistance, being highly resistant against cephalosporins, fluoroquinolones, aminoglycosides but sensitive to carbapenems and polymyxins.

KO809: From Site 3, recovered from Terg0 agar and displayed phenotypic resistance against only fluoroquinolones. This isolate was extremely tolerant to Triclosan, displaying an MIC of >1024mg/L.

KO880: From Site R, recovered from Terg0 agar and was phenotypically sensitive to every antibiotic, biocide and heavy metal tested. This isolate was sequenced for use as a sensitive control strain for comparison.

Table 16. Resistance profiles of eight K. oxytoca isolates

K. oxytoca		Class	Cephalosporins		Fluoroquinolones		Aminoglycosides		Carbapenems		Polymyxin	Disinfectants			Heavy Metal
		Antimicrobial	**FEP**	**CAZ**	**CIP**	**LEV**	**GEN**	**TOB**	**MER**	**IMI**	**COL**	**CHL**	**TRI**	**BNZ**	**$AgNO_3$**
		ECOFF	0.008-0.125	0.032-0.5	0.008-0.125	0.032-0.25	0.25-2	0.25-2	0.064-1	0.064-1	0.125-2	64	0.25	32	32
		Breakpoint	4	4	0.5	1	2	2	8	4	2	-	-	-	-
Isolate	**Site**	**Plated from**													
356	**1**	BileV+	>1024	>1024	64	16	512	64	0.5	0.5	0.0313	>64	0.5	256	>64
459	**2**	BileV+	0.015625	0.25	0.015625	<0.125	0.5	0.5	0.015625	0.5	0.125	2	512	16	16
480	**2**	Terg0	1	1024	0.5	0.5	1024	8	0.125	1	0.25	8	0.25	16	16
488	**2**	Terg0	0.5	128	0.5	2	128	8	0.125	0.5	0.125	4	0.125	8	>64
611	**3**	TergC+	1024	1024	16	4	256	64	4	0.5	0.125	32	1	16	4
618	**3**	TergC+	>64	>64	32	8	>64	32	2	0.5	0.125	16	4	16	4
623	**3**	TergC+	32	512	16	8	64	16	0.125	0.5	0.0625	32	0.5	8	4
809	**3**	Terg0	0.5	0.5	2	1	1	0.5	0.5	0.5	<0.125	16	>1024	16	16
880	**4**	Terg0	0.5	0.25	0.015625	0.015625	1	0.125	0.0625	0.25	0.125	<0.25	0.25	8	2

The minimum inhibitory concentrations, in mg/L, were determined for K. oxytoca isolates isolated from wastewater from four different sites at one hospital: Site 1: Outpatients; Site 2: Emergency Site 3: Oncology ; and Site 4: Reference site. Isolates were recovered from various selective media: Tergitol-7 without additional antibiotics Terg0; Tergitol-7 with 1mg/L of ciprofloxacin TergC+ and Bile aesculin with 4mg/L of vancomycin BileV+. Nine different antibiotics: cefepime(FEP), ceftazidime (CAZ), ciprofloxacin (CIP), levofloxacin (LEV), gentamicin (GEN), tobramycin (TOB), meropenem (MER), imipenem (IMI), colistin (COL) and 4 other antimicrobials: chlorhexidine (CHL), triclosan (TRI), benzalkonium chloride (BNZ), silver nitrate ($AgNO_3$) were used. Results were compared to the breakpoint and Epidemiological cut-off values, displayed in the table as mg/L, from the European Committee on Antimicrobial Susceptibility Testing or in the case of biocides and heavy metals, determined using the ECOFFFinderXL2010.xlsm tool (https://clsi.org/meetings/microbiology/ecoffinder/). Red indicates resistance against the tested compound.

4.1.2 Extended resistance profiles of K. oxytoca isolates were determined by disc diffusion

To extend the resistance profile of the sequenced isolates, disc diffusion assays were performed according to the EUCAST methods [Table 17] [118]. The disc diffusion method, where a standardised inoculum of an organism is grown in the presence of antibiotic impregnated discs, is the standard method for analysing antibiotic susceptibility profile in a diagnostic laboratory [194]. This method is widely used and is advantageous over MIC broth dilution methods as it is a quick way to visualise and test a large panel of antibiotics and to identify resistance against combined drug therapies, such as sulfamethoxazole-trimethoprim and piperacillin-tazobactam. Additionally, disc diffusion also allows for the exploration of other resistance mechanisms such as the visual detection of ESBL expression. To extend the number of antibiotics tested against, a of 15 antibiotics were tested across over six families of antibiotics. Where available, antibiotics tested within Chapter 3 were tested again to check for discrepancies. Disc diffusion results correlated well with the results from the broth dilution method for cefepime, ceftazidime, imipenem, meropenem, gentamicin and ciprofloxacin. Isolates were tested against an additional aminoglycoside, amikacin, of which all isolates were sensitive against, including those resistant to gentamicin. Combined antibiotic therapies were tested including piperacillin/tazobactam, an antibiotic combination given intravenously to treat serious infections [195] and sulfamethoxazole/trimethoprim, an oral combination to treat broad spectrum of Gram-positive and Gram-negative infections [196]. Isolates resistant to beta lactam, cephalosporin antibiotics and also the fluoroquinolone, ciprofloxacin (KO356, KO480, KO488, KO611 and KO623), were also resistant against the sulfamethoxazole/trimethoprim combination. Piperacillin/tazobactam resistance was observed in the highly multidrug resistant organisms KO356, KO611, KO623 and resistance was present in the generally sensitive isolate KO809 [Table 17].

Table 17. Disc sensitivity against K. oxytoca isolated from wastewater

Antibiotic Class	Antibiotic	Concentration (µg)	EUCAST		*Klebsiella oxytoca* isolate															
			S≥	R≤	356		459		480		488		611		623		809		880	
Cephalosporins	FEP	30	27	24	26	I	40	S	24	R	24	R	25	I	24	R	36	S	40	S
	COX	5	20	17	19	I	34	S	19	I	19	I	16	R	11	R	33	S	34	S
	CZD	10	22	19	0	R	35	S	9	R	9	R	0	R	0	R	29	S	38	S
Penicillins	AMC	30	14	14	0	R	29	S	14	R	13	R	0	R	0	R	11	R	26	S
	PTZ	36	20	17	11	R	32	S	24	S	26	S	0	R	0	R	0	R	28	S
Carbapenems	IPM	10	22	17	39	S	33	S	34	S	35	S	36	S	37	S	34	S	34	S
	MEM	10	22	16	37	S	34	S	36	S	35	S	34	S	34	S	37	S	36	S
Fluoroquinolones	CIP	5	25	22	16	R	37	S	24	I	24	I	16	R	12	R	24	I	44	S
Aminoglycosides	GMN	10	17	17	0	R	24	S	0	R	0	R	0	R	0	R	26	S	26	S
	AKN	30	18	18	20	S	26	S	26	S	27	S	21	S	22	S	27	S	26	S
Miscellaneous	NFE	100	11	11	12	S	26	S	26	S	29	S	24	S	24	S	24	S	42	S
	CHL	30	17	17	21	S	35	S	33	S	34	S	0	R	0	R	30	S	32	S
	TMP	5	15	15	0	R	34	S	0	R	0	R	0	R	0	R	31	S	40	S
	SXT	25	14	11	0	R	39	S	0	R	0	R	0	R	0	R	37	S	42	S
	TGC	15	18	18	26	S	26	S	27	S	29	S	24	S	24	S	25	S	24	S

K. oxytoca isolates (n=8) selected from wastewater were tested with disc sensitivity against 15 antibiotics following EUCAST's methods [118]. Numbers represent measured disc diameters in millimetres. Antibiotic abbreviations as written on discs: Cefepime (FEP); Cefotaxime (COX); Ceftazidime (CZD); Amoxicillin + Clavulanic acid (AMC); Piperacillin + Tazobactam (PTZ); Imipenem (IMP); Meropenem (MEM), Ciprofloxacin (CIP); Gentamicin (GMN); Amikacin (AKN); Nitrofurantoin (NFE); Chloramphenicol (CHL); Trimethoprim (TMP); Sulfamethoxazole + Trimethoprim (SXT) and Tigecycline (TGC).

Extended-spectrum β-lactamases (ESBLs) are plasmid-mediated enzymes able to hydrolyse 3rd generation cephalosporins and their production by bacteria severely limits treatment options, posing a current, major challenge in healthcare [197, 198]. Clinical methods are able to quickly detect the presence of ESBL producing organisms through variations of a disc diffusion method from guidelines set out by the Clinical Standards Institute or EUCAST [197]. These methods utilise the fact that *Enterobacterales* with inducible β-lactamase expression, are enhanced in the presence of particular, inducing compounds such as clavulanic acid. In other words, in the presence of clavulanic acid, chromosomal β-lactamase activity is increased in some strains of bacteria, limiting their treatment options in a clinical setting. In order to determine if the cephalosporin resistant *K. oxytoca* isolates from produce ESBLs, a disc diffusion assay was prepared as indicated in Figure 30. To test if the *K. oxytoca* isolates of interest would be detectable as ESBLs with phenotypic methods, imipenem (10 IU), augmentin (amoxicillin 20 IU and clavulanic acid 10 IU) and a cephalosporin, in this case cefotaxime (COX) (5 IU), were used with EUCAST's methodology. Imipenem, a carbapenem antibiotic, was included to detect for and exclude the action of any carbapenemases, whose presence is associated with cross resistance against cephalosporins [199]. Based on the phenotypical results KO356, KO480 and KO488 appear to harbour inducible ESBLS, indicated by a flattening of the zone of inhibition towards the COX disc [Figure 27].

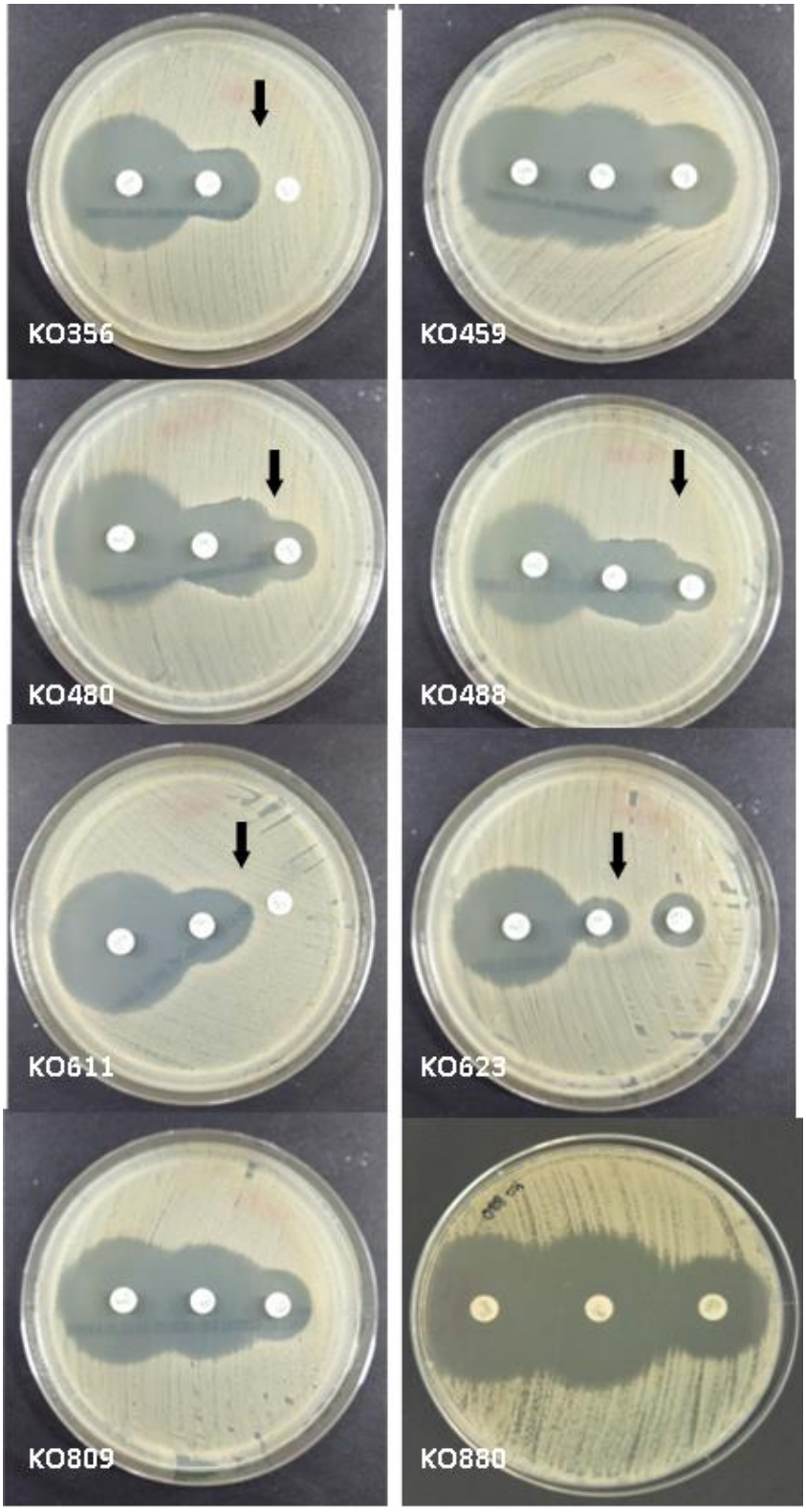

Figure 27. Detection of ESBL producing K. oxytoca isolates.
Disc sensitivity tests looks for extended spectrum β-lactamase production, indicated by flattening of the zone of inhibition towards the cefotaxime (COX) disc. K. oxytoca isolates (n=8) selected from wastewater were tested for phenotypic presence of extended β-lactamase using antibiotic discs: Imipenem (10 IU), COX (5 IU) and amoxycillin and clavulanic acid (AMC) (30 IU) (Left to right). Black arrows indicate presumptive ESBL producing isolates.

4.1.3 Genome analysis and assembly statistics

The genotypic resistance profiles of the eight isolates were compared to the observed phenotypic resistance profiles by analysis of their whole genome sequences. As previously mentioned, *K. oxytoca* is an emerging pathogen and thus, in comparison to pathogens such as *K. pneumoniae*, there exists relatively little data in the worldwide database for sequence analysis. This is exemplified by the fact that as of December 2022, there were 52 completed *K. oxytoca* genomes compared to 2000 completed *K. pneumoniae* submitted and available on the NCBI database [162].

WGS of the eight *K. oxytoca* isolates had a total genome length averaging 6,456,074 bp ranging from 6,071,209 to 6,751,689 bp. For reference, according to NCBI, the length refers to ungapped assembly length and acceptable sizes for bacterial sequences should be between 100,000 – 15,000,000 bp [162]. This consisted of an average of 230 contigs and an average GC content of 55.21%. The number of contigs ranged from 147- 322, lower contig numbers indicate higher quality of sequencing by indicating the number of sequenced DNA fragments present. Overall, the assembly statistics indicated quality sequence reads with low contig numbers and long contigs.

Table 18. Genomic assembly statistics of K. oxytoca (n=80) whole genome sequences isolated from hospital wastewater

Strain	Total length	GC %	Contigs	Largest contig (bp)
KO356	6751689	55.15	231	234339
KO459	6549794	55.45	276	367258
KO480	6478412	55.23	268	406723
KO488	6480982	55.24	322	296726
KO611	6384875	54.69	185	432055
KO623	6387516	54.69	206	544879
KO809	6544116	55.64	207	268054
KO880	6071209	55.59	147	339617

4.2 MLST and Phylogeny

4.2.1 The 8 K. oxytoca isolates belonged to five known and two unknown sequence types

Multilocus sequence typing (MLST) is a method of characterising isolates within a bacterial species by typing seven, well-conserved house-keeping genes *gapA, infB, mdh, pgi, phoE, rpoB and tonB* and comparing the allelic profiles to global databases [200]. From this, a sequence type (ST) is assigned, allowing isolates to be classified, identified, and used to identify evolutionary relationships [201]. In this study, STs was investigated for the eight sequenced isolates using the PubMLST database [200]. A total of five known STs were identified in six isolates: ST43, ST58, ST131, ST184 and ST216 [Table 19]. All five of these STs were first identified and curated in Canada between 2014-2020 but as of December 2022, there is no further information or publications available on the origin of any of these strains. Two isolates did not match any STs in the current 2022 PubMLST database, both matched closest with ST214, an unpublished sequence type, assigned to a *K. pneumoniae* isolate. The data suggests KO480 and KO488 is representative of new, uncommon, and unpublished sequence types. On completion of this project, the novel STs will be submitted to the database at PubMLST (https//pubmlst.org/).

Table 19. Sequence types of K. oxytoca isolated from hospital wastewater

Strain	*gapA*	*infB*	*mdh*	*pgi*	*phoE*	*rpoB*	*tonB*	MLST
KO356	3	8	41	43	51	6	47	131
KO459	4	6	26	10	46	5	6	184
KO480	6	6	19	10	46	5	16?	Unknown Closest match 214
KO488	6	6	19	10	46	5	16?	Unknown Closest match 214
KO611	1	2	2	1	4	1	2	58
KO623	1	2	2	1	4	1	2	58
KO809	3	5	21	20	11	6	20	43
KO880	4	6	19	10	46	24	31	216

Multilocus sequence types (MLST) were determined using PubMLST and MLST software (T. Seeman, https://github.com/tseemann/mlst).“?” indicates partial matches to a known allele.

4.2.2 Pangenome analysis

Isolates from the same species will contain a similar set of genes, referred to as the core genome, and will also contain a unique fingerprint of accessory genomes. Pangenome analysis can schematically represent the presence and absence of genes between samples, visually displaying the repertoire of genes to compare phylogeny. Pangenome consists of the core genes, genes present in all the tested isolates; and accessory genomes consisting of shell genes, genes present on two or more strains and cloud genes, genes only seen in a single strain. Amongst the 8 isolates, a total of 12932 genes were clustered; 3217 were core genes, representing 24.9% of the genomes; 3218 were cloud genes, representing 24.9% of the genomes and the remaining majority, 6497 were shell genes, representing 50.2% of the genomes. pangenome visualisation further revealed the evolutionary divergence of the 8 *K. oxytoca* isolates [Figure 28].

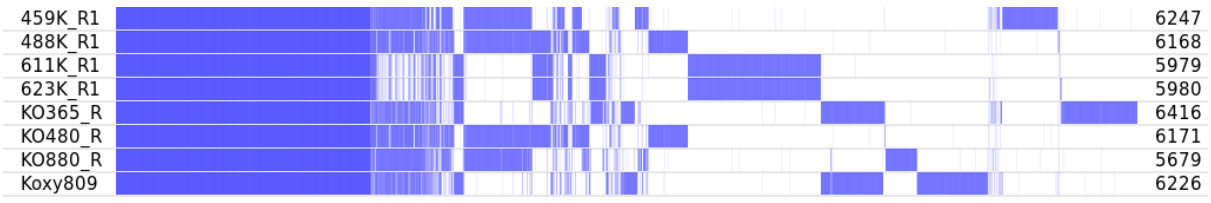

Figure 28. Comparison of core and accessory clusters of the eight K. oxytoca isolated from wastewater

Visualisation of pangenome-analysis of 8 K. oxytoca isolates. This schematic representation shows the presence (blue) or absence (blank) of genes between the isolates. Reading from left to right, the left indicates areas of high conserved core genomes (represented as darker blue) moving towards the right to show accessory genomes (light blue). This pangenome analysis revealed the evolutionary divergence within the sequenced isolates. Analysis was conducted using Roary V1.12.2 using the default parameters (T. Seemann, https://github.com/sanger-pathogens/Roary/blob/master/ contrib/roary2svg/roary2svg. Pl).

Although phenotypically, isolates KO480 and KO488 as well as KO611 and KO623 appeared to be almost identical, pangenome analysis was able to discern each pair as being clonally linked but not identical [Figure 28]. This shows differences between each pair especially towards the accessory genomes and is further demonstrated by Figure 29.

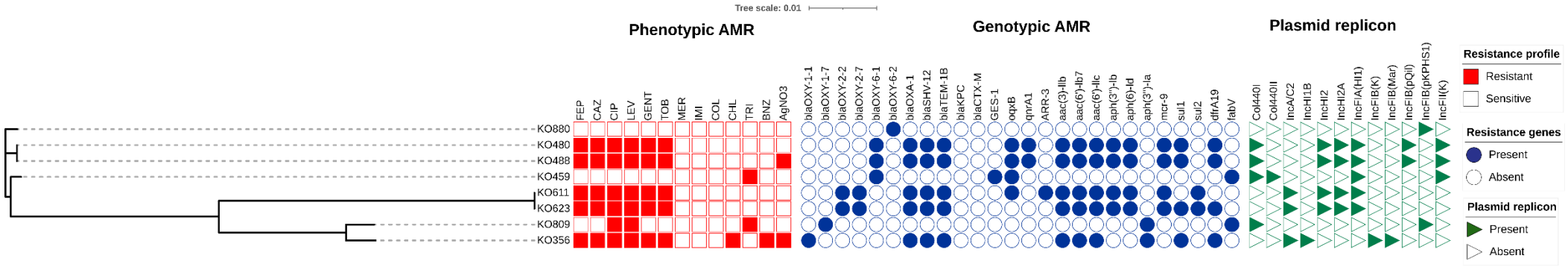

Figure 29. Phylogeny analysis of K. oxytoca isolates from hospital wastewater

Visualisation of the phylogenetic tree was carried out using iTOL [157]. Columns represent resistance phenotype: resistant (red squares) and sensitive (empty squares), resistance genotypes: presence (blue circles) and absence (empty circles) and predominant plasmid replicon types: presence (green triangles) and absent (empty triangles). Antibiotics tested were cefepime (FEP), ceftazidime (CAZ), ciprofloxacin (CIP), levofloxacin (LEV), gentamicin (GENT), tobramycin (TOB), meropenem (MER), imipenem (IMI) and colistin (COL). Genes $bla_{oxy1\text{-}1}$, $bla_{oxy1\text{-}7}$, $bla_{oxy\text{-}2\text{-}7}$, $bla_{oxy\text{-}6\text{-}1}$, $bla_{oxy\text{-}6\text{-}2}$, $bla_{OXA\text{-}1}$, $bla_{SHV\text{-}12}$, $bla_{TEM\text{-}1B}$, GES-1, confer resistance against cephalosporins in ESBLs; oqxB, qnrA1 confer resistance against fluoroquinolones; ARR-3, aac(3)-llb, aac(6')lb7, aph(6')-ld, aph(3'')-la, confer resistance against aminoglycosides; mcr-9 confers resistance against polymyxin, sul1, sul2 confers resistance against sulfonamides, dfrA19 confers resistance against triclosan and fabV confers resistance against triclosan.

4.3 *K. oxytoca* harbours multiple resistance genes

Analysis of *K. oxytoca* resistome revealed a plethora of AMR genes using a variety of online databases such as ResFinder, CARD-RGI as well as from the pipelines, within TORMES and Prokka. Data was visualised using iTOL [157] as summarised in [Figure 29].

4.3.1 Phenotypic cephalosporin resistance can be explained by presence of cephalosporin resistance genes

Resistance against 3rd generation cephalosporins is a currently a huge concern within *Enterobacterales* in healthcare settings [202, 203]. Here we report a total of 62 isolates, representing 70% of tested *K. oxytoca* isolates resistant against a 3rd generation cephalosporin, ceftazidime. Of these isolates, 96.7% were isolated from the three hospital sites. Compared to 3.2% from the reference site. Resistance against cephalosporins is most commonly due to plasmid mediated ESBLs (*bla*$_{\text{CTX-M}}$, *bla*$_{\text{TEM}}$ and *bla*$_{\text{SHV}}$) and AmpC beta lactamase (*bla*$_{\text{CMY-2}}$). *Bla*$_{\text{SHV}}$ and *bla*$_{\text{TEM}}$ were present amongst 5 MDR isolates, KO356, KO480, KO488, KO611 and KO623; *bla*$_{OXA}$ was carried by 3 isolates, KO356, KO611 and KO623. It was interesting to note, isolate KO611 was highly resistant against both FEP and CAZ (FEP MIC 1024mg/L ; CAZ MIC 1024mg/L) and carried 5 cephalosporin resistant genes; *bla*$_{\text{OXY-2-2}}$, *bla*$_{\text{OXY-2-7}}$, *bla*$_{\text{OXA}}$, *bla*$_{\text{SHV-12}}$ and *bla*$_{\text{TEM-1B}}$ in contrast KO356 which was also highly resistant (FEP MIC >1024mg/L ; CAZ MIC >1024mg/L), only carried *bla*$_{\text{OXA}}$, *bla*$_{\text{SHV-12}}$ and *bla*$_{\text{TEM-1B}}$. Isolates KO459, KO809 and KO880 were cephalosporin sensitive and only carried *bla*$_{\text{OXY-1-7}}$, *bla*$_{\text{OXY6-1}}$ or *bla*$_{\text{OXY-6-2}}$.

4.3.2 GyrA and ParC mutations were detected in the fluoroquinolone resistant isolates

Fluoroquinolones, such as ciprofloxacin, inhibit topoisomerase II, which is needed for supercoiling [57]. Enzymes DNA gyrase and topoisomerase IV, comprised of *gyrA/gyrB* and *parC/parB*, are also essential for supercoiling; thus, resistance in Gram-negative organisms is often associated with mutations in *gyrA/gyrB* or *parC/parB* [57, 58]. In this study, assessment of these proteins revealed numerous mutations which may be contributing to the observed fluoroquinolone resistance of the sequenced *K. oxytoca*. Alignment of the DNA gyrase, GyrA [Figure 30], revealed two mutations of interest. A Thr83IIe mutation was observed in the four isolates displaying MICs>2mg/L for ciprofloxacin. This position is frequently associated with mutations which give rise to fluoroquinolone resistance [58]. In other species of Enterobacteriaceae, the amino acid commonly found at position 83 is

serine, however in *K. oxytoca*, the threonine amino acid is found instead [204]. In *K. pneumoniae*, a substitution of serine to isoleucine at this position has been associated with low levels of fluoroquinolone resistance [58], with other studies showing an additional mutation in codon 8 are needed for higher levels of fluoroquinolone resistance [204]. In this study, although higher levels of ciprofloxacin resistance were observed in four isolates, no mutations were observed at position 87, however, a Gly409A1a mutation was identified in these isolates. Currently, this mutation has not been reported or characterised as such, it is unknown if it plays a role in increased ciprofloxacin or levofloxacin resistance.

A

	MIC (mg/L)							
Antibiotic	KO809	KO880	KO459	KO480	KO488	KO356	KO623	KO611
Ciprofloxacin	2	<0.125	<0.125	0.5	0.5	64	16	16
Levofloxacin	1	<0.125	<0.125	0.5	2	16	8	4

B GyrA alignment

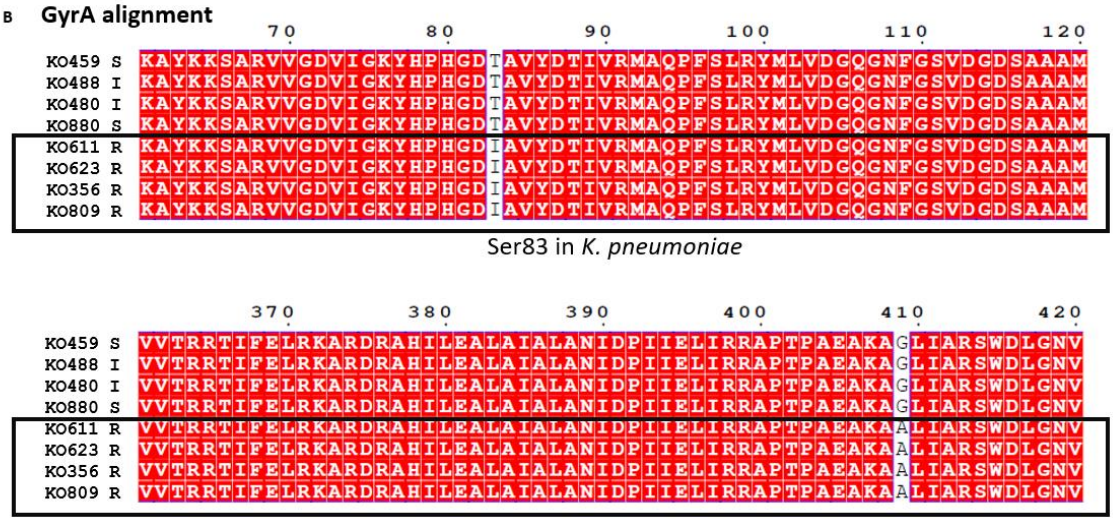

Figure 30. Multiple sequence alignment of gyrA

(A) Fluoroquinolone resistance profile of sequenced K. oxytoca isolates. (B). Multiple sequence alignment of the DNA gyrase GyrA mutation containing section.

In addition to GyrA, a multiple sequence alignment of GyrB also revealed a number of mutations [Figure 31]. Unlike GyrA, mutations within the GyrB subunits are not frequently associated with fluoroquinolone resistance, as this subunit is not a primary target of quinolones [205, 206]. Nonetheless, multiple conserved and non-conserved mutations are present, many of which here only found in the fluoroquinolone resistant isolates, as such may play a role in resistance. The most striking non-conserved mutation is the Arg688His mutation found in the fluoroquinolone resistant isolates only [Figure 31].

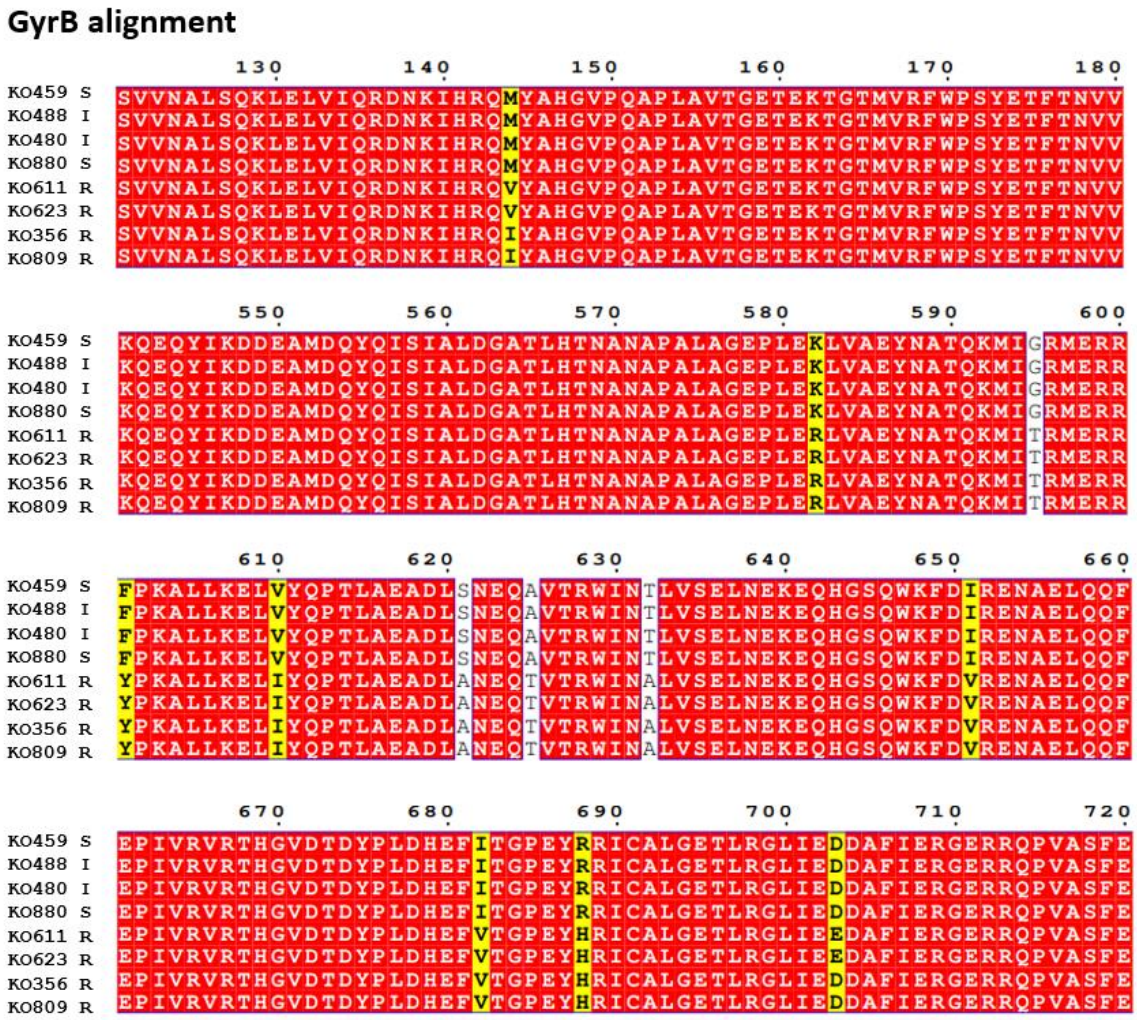

Figure 31. Multiple sequence alignment of the DNA gyrase GyrB sections illustrating numerous mutations in the protein.

Like DNA gyrase, topoisomerase IV is also composed of two subunits, ParC which is homologous to GyrA and ParE, homologous to GyrB [205]. Mutations within these are highly associated with increased fluoroquinolone resistance, with ParC in combination with GyrA mutations conferring high fluoroquinolone resistance [205]. In this study, numerous thus far uncharacterised mutations were identified in ParC [Figure 32]. Of particular interest, is the Ala467Ser mutation present in KO356, which was observed to have high resistance against ciprofloxacin (MIC 64mg/L). In addition to other conserved mutations, a non-conserved mutation, Thr246Ala, may also play a role in the increased ciprofloxacin resistance observed for the four *K. oxytoca* isolates.



Figure 32. Multiple sequence alignment of the topoisomerase IV, ParC, sections containing mutations.

Assessment of the ParE topoisomerase IV subunit revealed additional conserved mutations [Figure 33] are unlikely to contribute to fluroquinolone resistance. However, studies on *E. coli* have revealed ParE mutations, at position 458 and 529 which have been found in ciprofloxacin resistant isolates [207]. As such, further research needs to be carried out to assess the mutations found in this study and their potential effect on fluoroquinolone resistance in *K. oxytoca*.

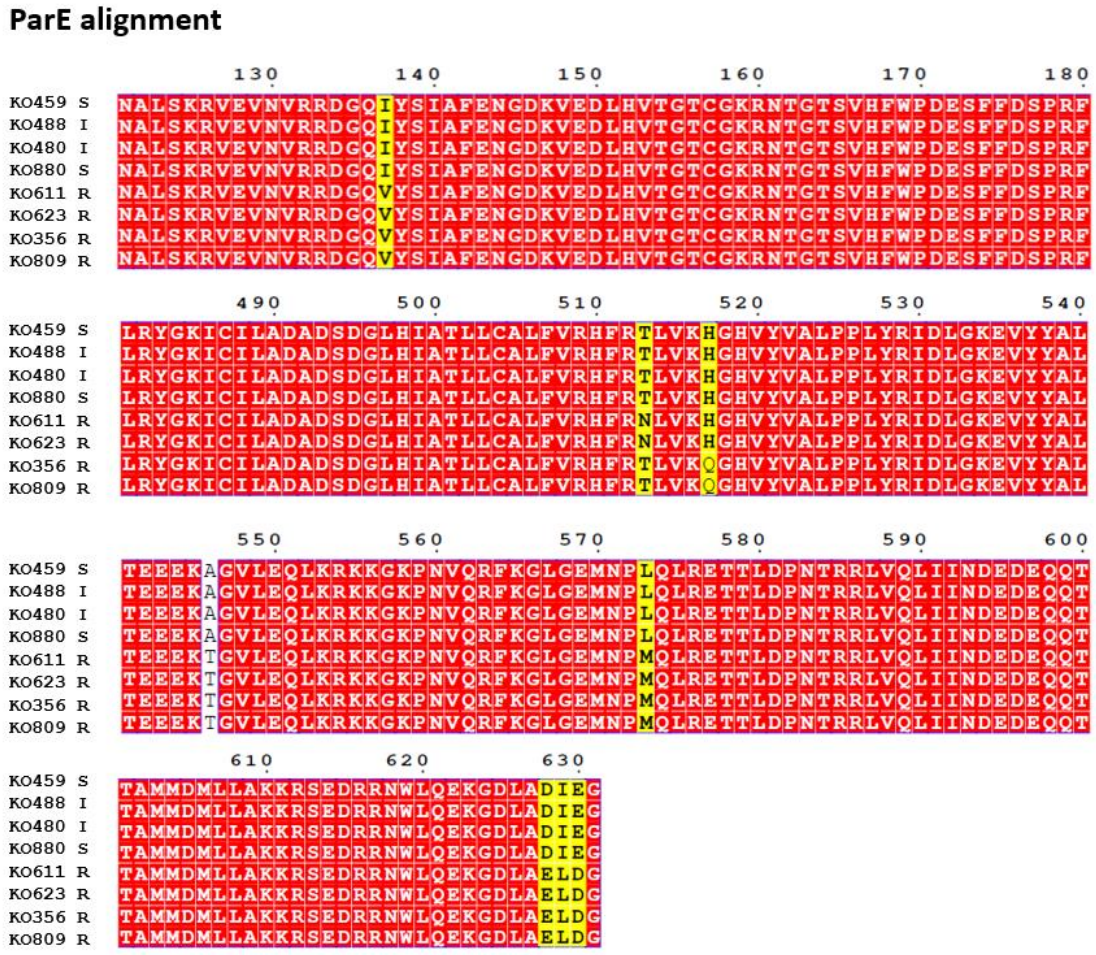

Figure 33. Multiple sequence alignment of the topoisomerase IV, ParE, sections containing mutations.

Finally, drug efflux pumps from the RND family such as AcrAB-TolC are also recognised as drivers of resistance against fluroquinolones [208], and may also be contributing to the fluoroquinolone resistance observed in the *K. oxytoca* isolates assessed here.

4.3.4 All isolates are colistin sensitive despite the presence of mcr-9

Genes conferring resistance to polymyxins such as colistin was not expected in these isolates as all of the isolates were colistin sensitive. The most common genes associated with polymyxin resistance in Gram-negative organisms include the mobile colistin resistance *(mcr)* genes [209]. Resistance against colistin is usually associated with the modification of bacterial lipopolysaccharide due to systems such as the PhoPQ and MgrB system. In *K. pneumoniae*, resistance is commonly associated with inactivation due to mutations or insertions within *mgrB* [210]. Furthermore, point mutations in the *pmrB* gene has been associated with cross resistance between chlorhexidine and colistin [211]. Unexpectedly, *mcr-9* was found in 4 isolates: KO480, KO488, KO611 and KO623. It is interesting to note

these same isolates also carried the IncHI2 and IncHI2A plasmids. This supports studies where *mcr* genes have been found on IncHI2 plasmids and worryingly, its transmission within healthcare on these plasmids, has been recently demonstrated [212, 213].

4.4 The *K. oxytoca* isolates carried biocide resistance determinants

The isolates were tested for resistance against biocides: silver nitrate, triclosan and chlorhexidine. Isolates KO459 and KO809 had a very high level of resistance against triclosan (MIC 512mg/L and >1024mg/L). Sequence analysis was performed, and resistance determinants were searched for using the Bacmet database to find whether these isolates carried any genes conferring resistance against biocides [Appendix 11].

4.4.1 Triclosan resistant organisms carried fabV from V. cholera

Triclosan is a widely used biocide, prevalent throughout healthcare and household products and acts by binding to bacterial enoyl-acyl protein reductase enzyme to block lipid biosynthesis [214]. Triclosan resistance is currently associated with functional gene mutations and changes in efflux pump action. Mechanisms of triclosan resistance are commonly reported in other Gram-negative organisms such as *E. coli* and *K. pneumoniae* and include mutations in regulators such as *fabI, acrB, acrF and marA* resulting in changes of activity and changes to levels of expression [97]. Isolates KO459 and KO809 displayed extremely high MIC values against triclosan (MIC 512 mg/L and >1024mg/L respectively) prompting the search through BacMet for potential genes that may confer resistance [184]. Unlike the six triclosan sensitive strains, both isolates KO459 and KO809 contained the *fabV* gene known to confer triclosan resistance in other species such as *V. cholera* and *P. aeruginosa* [215]. The FabV protein, is an enoyl-acyl carrier protein reductase enzyme, involved in fatty acid synthesis and deletion of *fabV* gene has been shown to decrease bacterial mobility, decreased fatty acid synthesis, bacterial swarming, reduced pathogenicity and cause triclosan sensitivity within *P. aeruginosa* [215]. Both FabV proteins matched 100% to a trans-2-enoyl-CoA reductase family protein from *Enterobacterales* [Chain A, Enoyl-reductase NADH, *Vibrio fischeri* ES114 Accession number 5X10.A]. Both isolates align perfectly to each other but were distinct to the commonly referenced FabV from *P. aeruginosa* [84], accession number Q9HZP8 [Appendix 9] and *V. cholera*, accession number *WP_000584512.1*

4.4.2 Silver Nitrate determinants silP and silE were found in all isolates

Silver nitrate resistance was phenotypically observed in KO356 and KO488 (MIC >64mg/L) and common resistance determinants against silver and other heavy metals were investigated. Appendix 11 displays all searched determinants currently known to affect silver resistance. Notably, all sequenced isolates carried genes for the Silver Exporting P-type ATPase *silP* and 2 variants of Silver Binding Protein SilE [Appendix 11]. The role of the SilE protein in silver resistance is not clear but has been shown to be a crucial aspect for phenotypic resistance [216]. The mechanism of silver resistance in KO356 and KO488 are unknown as the *sil* genes found were similar amongst all isolates, sensitive or resistant to silver nitrate. This is an important area for further investigations.

4.5 Mobile Genetic Elements

4.5.1 K. oxytoca contains 12 plasmids from various other Gram-negative organisms

Plasmids are circular, extrachromosomal bacterial DNA able to carry genes conferring antibiotic resistance including to third generation cephalosporins [217]. Plasmid replicon typing allows the classification and identification of plasmids [218]. The eight sequenced isolates contained a total of 12 plasmid replicon types with 95.27-100% identity matched to CGE's PlasmidFinder database. These 12 plasmids were predominantly from incompatibility groups (Inc), a large, highly promiscuous plasmid group commonly found amongst *Enterobacterales* from a wide variety of sources [217]. The isolate belonging to the ST131 family carried *IncA/C2, IncFIB(K), IncFIB(Mar),* isolates belonging to the ST184 family carried *col440I, IncFIA(HI1)* and *IncFII(k)*. ST58 family carried *IncA/C2, IncHI2/2A, ST43* carried *Col440I* and *IncFIB(pKPHS1), ST216* carried *IncFIA(HI1)* and the unknown ST both carried *IncHI2/2A, Col440I, IncFIA(HI1), IncFII(k)* and *IncFIB(pQil)* [Table 20]. PlasmidFinder matched 75% (n=9) of the plasmids with plasmids from *K. pneumoniae* in the database. Isolates carrying *IncHI2/2A* matched 100% with a plasmid isolated from *S. marcescens* and *IncFIA(HI1)* was aligned with a plasmid from *S. typhi* in the database. The presence of these plasmids explains some of the resistance genes present on the eight isolates, Chapter 4.3.1 noted the high level of cephalosporin resistance and presence of bla_{OXA} on isolates KO356, KO611 and KO623. The bla_{OXA} gene is carried on plasmid *IncA/C2*, which was present in those isolates.

Table 20. Presence of different plasmid replicon types among 8 sequenced K. oxytoca isolates

		Isolate and MLST							
Plasmid	**Plasmid information**	**KO356**	**KO459**	**KO480**	**KO488**	**KO611**	**KO623**	**KO809**	**KO880**
		ST131	**ST84**	**ST Unknown**	**ST Unknown**	**ST58**	**ST58**	**ST43**	**ST216**
Col440I	*K. pneumoniae FDAARGOS_440 unnamed 1*		96.49	95.61	95.61			96.5	
Col440II	*K. pneumoniae strain FDAARGOS_440 unnamed 2*		98.94						
IncHI1B	*K. pneumoniae plasmid pNDM-MAR*	99.82							
IncHI2	*S. marcescens plasmid R478*			100	100	100	100		
IncHI2A	*S. marcescens plasmid R478*			100	100	100	100		
IncA/C2	*K. pneumoniae strain Kp7 plasmid pNDM-KN*	100				100	100		
IncFIA(HI1)	*S. typhi R27 plasmid*		96.13	96.13	96.13				96.13
IncFIB(K)	*K. pneumoniae strain ST258 plasmid kPN-IT*	99.11							
IncFIB(Mar)	*K. pneumoniae plasmid pNDM-MAR*	99.54							
IncFIB(pQil)	*K. pneumonieae strain ST258 plasmid kpQIL-IT*			100	100				
IncFIB(pKPHS1)	*K. pneumoniae HS11286 plasmid kPHS1*							100	96.25
IncFII(K)	*K. pneumoniae MGH 78578 plasmid pKPN3*		95.95	95.27	95.27				

Percentage matched to plasmid replicon types among 8 sequenced K. oxytoca isolates using CGE's PlasmidFinder database.

4.6 Summary of sequence analysis of *Klebsiella oxytoca*

K. oxytoca is an emerging pathogen with a large genome, typically 6 million base pairs, has a genome with high plasticity, and as such, has a large potential for the dissemination of MDR genes [193]. Despite this, characterisation of *K. oxytoca* is poor and this exists as a large gap within literature [219]. One way to begin characterising and identifying the origin and evolution of bacterial isolates is through the assignment of multi-locus sequence types. In chapter 4, multi-locus sequence typing revealed 5 known and 2 unknown sequence types amongst the 8 sequenced *K. oxytoca* [Table 19] identified sequence types (STs) were ST43, ST58, ST131, ST184 and ST216 and although these STs are currently assigned within the

NCBI database, no further information or publications exist to reveal the origin of the strains. This is unsurprising when considering the lack of research into characterising emerging pathogens. Phenotypically, these isolates were almost identical and were sensitive against the tested biocides, sensitive against carbapenems, polymyxins and resistant against cephalosporins, fluoroquinolones and aminoglycosides. This study may potentially be the first to identify this sequence type. These two novel, unknown sequence types will be further reviewed, characterised in future studies to be submitted to the database in PubMLST [200].

Enterobacterales resistance against 3rd generation cephalosporins is currently a major concern within healthcare [202, 203]. Recently, a 2022 analysis found less than 10% of their hospital isolated *K. oxytoca* to be resistant against 3rd generation cephalosporins and attributed the resistance due to the presence of extended beta-lactamases bla_{TEM} and bla_{SHV} [127]. Here we report a total of 62 isolates, representing 70% of tested *K. oxytoca* isolates resistant against a 3rd generation cephalosporin, ceftazidime. Of these isolates, 96.7% were isolated from the three hospital sites, representing a significantly proportion than the 3.2% from the Reference site. Our current study confirms the presence of ESBL genes bla_{TEM} and $bla_{\text{SHV-12}}$ conferring resistance against 3rd generation cephalosporins in four resistant *K. oxytoca* isolates [Table 14].

Resistance against fluroquinolones in Gram-negative organisms are often associated with mutations in in *gyrA/gyrB* or *parC/parB* [57, 58]. Here we report mutations in *gyrA* within four sequenced isolates phenotypically resistant (MICs >2mg/L) against ciprofloxacin. A Gly409Ala mutation was observed at position 87, and has not been previously reported or characterised, as such, it is unknown if it plays a role in increased fluoroquinolone resistance. Multiple mutations in ParC were also present, of interest is the Ala467Ser mutation in isolate KO356 which had the highest level of ciprofloxacin resistance. Additional mutations were found on ParE, but further work is required to assess the effects these mutations have on fluoroquinolone resistance in *K. oxytoca*.

Triclosan is a widely used antimicrobial and disinfectant, present in healthcare and household products [97] but triclosan accumulation in the environment has been shown to reduce bacterial in vivo antibiotic sensitivity by 100-fold [220]. Furthermore, resistance against triclosan is associated with cross resistance with antibiotics in Gram-negative bacteria [22]. To date, no study has focused on triclosan resistance within environmental or emerging pathogens, which have potential to carry and disseminate resistance genes. As such, no ECOFF value exists for *K. oxytoca* against triclosan. This study found *K. oxytoca's* 95% ECOFF value against triclosan to be 0.25mg/L. A total of 37.8% *K. oxytoca* (n=10)

were resistant against triclosan and two isolates, were completely insensitive to triclosan as found in Chapter 3.4.6. Triclosan resistance is currently associated with functional gene mutations and changes in efflux pump action. In other *Enterobacterales* such as *E. coli* and *K. pneumoniae,* mechanisms of triclosan resistance are commonly due to mutations in *gyrA*, *fabI, acrB, acrF* and *marA,* resulting in changes to levels of expression [97]. Mutations in *gyrA* can lead to resistance or a decrease in susceptibility against triclosan, demonstrated in *E. coli* and *Salmonella spp.* [221] however, amongst these triclosan resistant *K. oxytoca* isolates, no mutations were found in *gyrA*. One common triclosan resistant determinant includes *fabV*, previously isolated from *P. aeruginosa* [84] and variants, such as *fabL* have been found in *B. subtilis*, *fabK* in *E. faecalis* and *fabV* in *V. cholerae* [84]. In this chapter, *fabV* genes were found in the two, highly triclosan resistant isolates. The *fabV* genes were 398 AA in length and when blasted with the NCBI database, aligned closely with the published *fabV* genes isolated from *P. aeruginosa* [Appendix 9] and *V. cholerae* [Appendix 10]. Additionally, unlike the other six sequenced *K. oxytoca* isolates, which were triclosan sensitive, the triclosan resistant strains contained an additional subunit of *acrB* [Appendix 8]. The genomically encoded *acrB* gene is well established efflux protein associated with AMR through upregulation or mutation [222, 223]. Further investigations are required to explore the significance and relationship of triclosan resistance with additional *acrB* subunits and the presence of *fabV* determinants within *K. oxytoca.*

Previous studies have demonstrated the ability for *K. oxytoca* to carry an array of virulence genes, but the presence of common virulence genes was not demonstrated within this study [126] [Appendix 7]. The exploration of *K. oxytoca*'s virulence genes were outside the scope of this study but the ability for *K. oxytoca* to acquire or disseminate virulence genes remains largely unknown and is worthwhile for future studies to investigate.

Plasmids are mobile genetic elements, capable of carrying resistance determinants and disseminating to other isolates [202, 224]. In Chapter 4.5.1, twelve plasmid vectors were identified amongst the eight sequenced *K. oxtoca* isolates. The twelve plasmids originated from *K. pneumoniae, S. typhi* and *S. marcescens,* clearly illustrating the worrying ability for emerging pathogens to acquire resistance mechanisms from other organisms. Many resistance determinants identified earlier are known to be carried on mobile plasmids, including the beta-lactamase *bla* genes. As noted earlier, the presence of these plasmids

explains some of the resistance genes present on the eight isolates; high levels of cephalosporin resistance can be explained by the presence of *bla*$_{\text{OXA}}$ carried on plasmid *IncA/C2* in KO356, KO611 and KO623.

As demonstrated, emerging pathogens such as *K. oxytoca* carry a large number of resistance determinants in selective environments and thus, have potential to be disseminators of resistance determinants to other species and organisms. The *K. oxytoca* complex is largely unstudied and future studies need to explore their disseminating potential. Studies should also explore mechanisms of biocide resistance determinants in emerging pathogens, such as the triclosan determinant *fabV* found in *K. oxytoca*.

Chapter 5: Discussion and future work

5.1 Discussion

The development and dissemination of antimicrobial resistance (AMR) is one of the most concerning issues currently facing society [186, 225]. Since the initial discovery of antibiotics in 1928, bacterial resistance rates have severely surpassed the rate of drug discovery [8, 23, 71, 125]. Bacteria are becoming increasingly resistant to antibiotics, so much so that rates of multi-drug resistance are developing at an alarming rate limiting the availability of drugs able to combat these infections[12]. It is not just antibiotic resistance rates that are concerning. Bacteria have been showing resistance against other antimicrobials including biocides such as disinfectants and heavy metals, which are used throughout hospitals and in everyday household products [96, 98, 226]. Resistance against biocides and heavy metals has been linked to cross-resistance and decreased sensitivity with antibiotics and linked to the development of multidrug resistance [220, 226].

The rising rate of antibiotic resistance is attributed to the abuse and overuse of antibiotics, thus in recent years, antimicrobial stewardship programmes have been implemented [12, 39]. To address this serious global threat, an action plan was endorsed by the WHO to help combat the issue of AMR and that plan included the need to "*strengthen knowledge through surveillance*" [41]. Furthermore, the need to coordinate and collaborate surveillance and antibiotic stewardship across human health, animal health, food, pharmaceutical and agricultural industries has led to the adaptation of a One Health plan, by the Australian Department of Health and Aged Care and the Department of Agriculture and Water Resources [33]. Surveillance of pathogens from wastewater has seen marked interest in the last 3 years due to the COVID-19 pandemic [145]. Wastewater surveillance as a public health tool, is a fast-evolving area with immense potential. It can serve as an early warning tool, or employed in this study, it can be used to obtain holistic data for an entire healthcare facility [146]. The increased interest in wastewater testing for public health transcends into surveillance of AMR markers, to search within particular geographical areas for genes that may indicate increased levels of resistance [147]. AMR in wastewater can be surveyed using phenotypic or genotypic methods. Researchers are moving away from traditional culture-based methods for the identification and profiling of bacteria, as laboratory-based methods are considered highly laborious and time-consuming [148]. While genomic methods are very powerful, they are most informative when used in conjunction with culturing and phenotypic analysis. There is a phenotypic vs genotypic discrepancy, the presence or absence of a gene does not necessarily correlate with AMR or sensitivity [163]. This is

demonstrated when observing colistin sensitive *K. oxytoca*, carrying mcr-9 genes, likely carried on the IncHI2 and IncHI2A plasmids (Chapter 4.3.4) and further demonstrated with a lack of correlation between silver sensitivity and resistance, despite the presence of common silver nitrate determinants *silP* and *silE* found in other Enterobacterales (Chapter 4.4.2).

It is not clear yet how wastewater environments may influence the dissemination of AMR genes however, hospital wastewater has a higher potential for antibiotic resistance [99, 100] when compared to urban wastewater. Furthermore, little is still known regarding healthcare wastewater environments and its ability to harbour or disseminate resistant ecological niches. In this study, a link between ecological niches and increased resistance phenotypes was observed, as discussed in Chapter 3 for Gram-negative populations *C. freundii, E. cloacae, E. coli, K. pneumoniae* and *K. oxytoca*: from three different hospital sies and one community wastewater site. The highest rate of resistance against cephalosporins, fluoroquinolones, carbapenems and aminoglycosides were from bacterial species isolated from Theatre/Emergency and Radiology/Oncology wastewater sites and these sites had an increased population of multidrug resistance when compared to the hospital's residential wastewater and a community wastewater site (Chapter 3.3). Conversely, the latter two sites contained almost entirely sensitive bacterial populations and did not harbour any multidrug resistant populations. Furthermore, through 16s sequencing, this study demonstrated that the sites with the highest multidrug resistant populations also had the highest levels of bacterial diversity. Our study supports the notion, ecological niches provide selective pressures that give rise to unique population diversity [110, 227-229]. This is consistent with literature where research demonstrates hospital wastewater harbours increased levels of antibiotic and multidrug resistance [108, 110]. Other studies have demonstrated different bacterial populations compared to different sites internally within a hospital or compared different hospitals [230, 231]. COVID-19 wastewater studies have also investigated bacterial biodiversity and found major phyla include *Actinobacteria, Firmicutes, Bacteroidetes and Proteobacteria* [147]. As demonstrated in Chapter 3. 2, by looking at bacterial diversity amongst different sites in one hospital, proportions of phyla are dependent on its environment. To the best of my knowledge, this is the first study to demonstrate bacterial diversity and multidrug resistance differences from sites receiving wastewater from different areas of a single, major metropolitan hospital. Microbiome diversity is affected by dispersal sources and selective pressures from the environmental conditions and future studies should investigate the differences in wastewater selective pressures within the same hospital that are giving rise to higher levels of multidrug resistance.

The development of antibiotic resistance is a normal, adaptive process of natural selection, to increase fitness and optimise species survival [28-30], however, the increasing rate of development and acquisition of resistance determinants against commonly used antibiotics such as cephalosporins, fluoroquinolones and aminoglycosides is extremely concerning. Currently, 3rd generation cephalosporins are considered the best available treatment against multidrug resistant organisms [1] but worryingly, this study demonstrates resistance against 3rd generation cephalosporins in an alarming number of isolates classified by the WHO as *Critical Priority 1* on the *Global Priority Pathogens List of Antibiotic Resistant Bacteria* [125]. Priority 1 organisms include the *Enterobacterales*, and as demonstrated in this study, *C. freundii* (96%), *E. coli* (58%), *E. cloacae* (88%) and *K. pneumoniae* (76%) were highly resistant or completely insensitive to ceftazidime, a 3rd generation cephalosporin, up to 1000 times above the EUCAST MIC. Furthermore, this study has also demonstrated ceftazidime resistance in an opportunistic pathogen, *K. oxytoca*. Although *K. pneumoniae* have been reported recently to be resistant against 3rd generation cephalosporin few studies have reported the same level of resistance in *K. oxytoca* [127]. Organisms considered environmental and opportunistic have become an area of high concern they often require more serious antibiotic therapies when treating infections caused by them [219]. These organisms, fit enough to survive the selective pressures within environments, may be more resilient compared to clinically acquired pathogens and may thus be able to contribute greatly to the dissemination of resistance genes [232]. Moreover, their interactions with other opportunistic organisms, their ability to develop and acquire resistance in complex environments and their role in the carrying and dissemination of resistance determinants is not certain [149].

K. oxytoca is a complex of opportunistic organisms with a large potential for the dissemination of MDR genes [126]. The *K. oxytoca* complex are becoming a problem within healthcare [131, 132] and infections by the *K. oxytoca* complex with drug or multidrug resistance are becoming more common [127]. Relatively few studies have focused on *K. oxytoca* however as it was highly prevalent within the hospital samples and observed to be resistant against a number of antibiotics assessed in this study (Chapter 3), it was chosen for further analysis. In-depth analysis and sequence typing using whole genome sequencing revealed the eight *K. oxytoca* isolates belonged to 5 sequence types and 2 unassigned sequence types. These 5 sequence types currently do not have any available literature regarding their origins or characterisations. These two novel, unknown sequence types will be further reviewed, characterised in future studies to be submitted to the database in PubMLST [200].

In this study, *K. oxytoca* resistant against 3rd generations was identified in isolates carrying ESBL genes *bla*$_{TEM}$ and *bla*$_{SHV-12}$. These findings support a recent study from an Australian hospital which found 23% of *K. oxytoca* to carry *bla*$_{TEM}$ and *bla*$_{SHV-12}$ [127]. The presence of these ESBL genes is significant as these are known to be carried with other genes encoding for resistance against other antibiotic classes including fluoroquinolones, aminoglycosides [233]. In this study, six of the eight sequenced isolates were fluoroquinolone resistant. Resistance against fluroquinolones in Gram-negative organisms are often associated with mutations in in *gyrA/gyrB* or *parC/parB* [57, 58] and well characterised in other *Enterobacterales* species [234]. Here we report mutations in GyrA within four sequenced isolates phenotypically resistant (MICs >2mg/L) against ciprofloxacin. A Gly409Ala mutation was observed at position 87 (Chapter 4.3.2; Figure 30), and has not been previously reported or characterised, as such, it is unknown if it plays a role in increased fluoroquinolone resistance. Multiple mutations in *ParC* were also present. Of interest is the Ala467Ser mutation in isolate KO356 which had the highest level of ciprofloxacin resistance (MIC=64mg/L, EUCAST Breakpoint=0.5mg/L; Chapter 4; Table 16). Additional mutations were found on *ParE*, but as these have not been previously explored in *Klebsiella spp.*, further work is required to assess the effects these mutations have on fluoroquinolone resistance within the species. This illustrates that although mechanisms of resistance may be well characterised in many other Gram-negative organisms, emerging pathogens such as *K. oxytoca* require further work to characterise resistance mechanisms.

An important aspect of this study included the investigation into the ECOFF values against common household biocides. Triclosan for instance, is a widely used antimicrobial and disinfectant, present in healthcare and household products [97] but its accumulation in the environment has been shown to reduce bacterial antibiotic sensitivity by 100 fold *in vivo* [220]. Furthermore, resistance against triclosan is associated with cross resistance with antibiotics in Gram-negative bacteria [22]. To date, no study has focused on triclosan resistance within environmental or emerging pathogens. previously, no ECOFF value existed for *K. oxytoca* against triclosan and for the first time, this study found the 95% ECOFF value against triclosan to be 0.25mg/L. Chapter 4 tested the minimum inhibitory concentrations against triclosan finding 37.8% of *K. oxytoca* were triclosan non-wildtype. To investigate the genetic component behind the triclosan resistance, two triclosan insensitive strains were sequenced. One common triclosan resistant determinant includes *fabV,* previously isolated from *P. aeruginosa* [84] and variants, such as *fabL* have been found in *B. subtilis*, *fabK* in *E. faecalis* and *fabV* in *V. cholerae*

[84]. To the best of my knowledge, this is the first study to show two *K. oxytoca* isolates carrying a *fabV* gene, matching with *fabV* genes isolated from *P. aeruginosa*.

This study illustrates the importance of culture-based methods for the phenotypic surveillance of multidrug resistant organisms and revealed its ability to show and study the differences in ecological niches. For instance, these results demonstrated for the first time, the presence of *mcr-9* in colistin sensitive *K. oxytoca*. Previous studies found *mcr-9* gene, attributing it's potential colistin resistance in *E. coli* and *Salmonella spp.* but recently studies found the *mcr-9* gene had no role in colistin resistance in either organism [163, 164]. The presence of *mcr-9* in colistin sensitive *K. oxytoca*. supports the notion that genomic information should be paired with phenotype.

In conclusion, this study brings to light the importance of incorporating phenotypic and genotypic methods into future surveillance of AMR development and spread. Evidence in this study shows reliance on the detection of resistance genes with purely genomic data does not give reliable indication regarding its level of phenotypic expression, further supporting the notion that genomic information should be paired with phenotype for more accurate AMR surveillance.

We have demonstrated that different areas from a single hospital site present different selective pressures which in turn, are associated with differences in phenotypic resistance levels, presence or absence of resistance genes and influences population diversity. Previously uncharacterised mutations within *gyrA/gyrB, parC/parE* genes were identified and may confer resistance to fluoroquinolone antibiotics. Finally, this study also presented an opportunistic pathogen, *K. oxytoca,* with complete resistance against a common, household biocide, triclosan, and for the first time, found the presence of *fabV* gene within these resistant strains.

5.2 Future work

The findings in this thesis have demonstrated that wastewater received from different areas within a major hospital contain highly different populations of resistant bacteria. This link was observed with *Enterobacterales* species *C. freundii, E. coli, E. cloaca*e, *K. pneumoniae* and *K. oxytoca*. Furthermore, this study showed different sites from one hospital contain vastly different population profiles. Further studies are needed to investigate the constituents in the wastewater from different sites within one hospital and how they select for and shape their ecological niches. This could be through largescale studies exploring the usage of drugs and disinfectants in hospital sites and may provide a clearer links to understanding how each hospital site influences

AMR populations in receiving environments. Such studies would generate data that may help better understand how selective pressures give rise to resistance and to inform changes which can slow down the dissemination and development of AMR.

A key finding from this project was the Gly409Ala mutations in GyrA in ciprofloxacin resistant *K. oxytoca* and the Ala467Ser mutation in a particular isolate with ciprofloxacin resistance 128 times above the breakpoint, these have not previously reported or characterised. Future work is required to assess the role these mutations may have on fluoroquinolone resistance in *Klebsiella spp*.

Since the completion of this study, the major hospital relocated to a new site and today, the same sampled sites no longer receive wastewater from the hospital. This provides a unique opportunity for future studies to assess these sites for changes in their bacterial profile and AMR populations, greatly towards better understanding the impact different selective pressures may have on ecological niches.

Chapter 6: Conclusion

This study investigated three different wastewater sites from one, major, metropolitan hospital. Phenotypic analysis of hospital wastewater was performed by searching for and isolating emerging pathogens using selective and differential media, Tergitol-7, Bile aesculin and *Pseudomonas* Agar, selected because of their ability to search for a variety of antimicrobial resistant organisms from the samples. These media were able to isolate viable opportunistic pathogens for further investigations into their resistance profiles. Resistance profiling, coupled with bacterial 16s, revealed each wastewater site harboured its own unique bacterial population, representing distinct ecological niches.

The use of phenotypic methods for the isolation of organisms from complex environments is a necessary and highly useful method for finding viable, resistant isolates. The combined use of selective/differential media with various genetic analysis techniques: Whole Genome Sequencing, 16s Microbial Population Profiling was shown to be an effective and powerful way to analyse and compare both the phenotypical and genotypical presentation of organisms. In this study, the most prevalent bacterial species isolated were *Pseudomonas spp., Klebsiella spp.*, and *Acinetobacter spp.*. A very large proportion of environmental or emerging pathogens were isolated. One such pathogen is the *Klebsiella oxytoca* complex, which in this study was shown to exist in very high proportions as multidrug resistant organisms. Organisms isolated from the Oncology/Radiology and Theatre/Outpatient/Emergency departments were highly resistant against cephalosporins, fluroquinolones and aminoglycosides when compared with the residential and the reference site. This distinct resistance/sensitive pattern amongst the different sites was observed in other organisms tested including *C. freundii, E. cloacae* and *K. pneumoniae*. This notion supports the widely accepted problems selective environments have on bacterial populations. Within the resistant *K. oxytoca*, various genes were found and attributable to phenotypic resistance including extended beta-lactamases $bla_{\text{CTX-M}}$, bla_{TEM} and bla_{SHV}. Another worrying gene of interest was carried was colistin resistant $mcr_{\text{-9}}$ gene, carried on colistin sensitive *K. oxytoca* strains. Although sensitive, an emerging pathogen carrying known resistance determinants against a last-line antibiotic in an environment prevalent with clinically pathogenic strains illustrates a grim and precarious situation.

In conclusion, this study further exemplifies the need for studies to focus not only on pathogenic organisms, but also on emerging pathogens which have potential to carry resistance determinants and themselves, be highly resistant. This study demonstrated the power of wastewater-based epidemiology as an additional tool for AMR surveillance through the combined use of phenotypic and genotypic methodologies.

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Chapter 8: Appendix

Appendix 1. Efflux Pump-Driven Antibiotic and Biocide Cross-Resistance in Pseudomonas aeruginosa Isolated from Different Ecological Niches: A Case Study in the Development of Multidrug Resistance in Environmental Hotspots

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Efflux Pump-Driven Antibiotic and Biocide Cross-Resistance in *Pseudomonas aeruginosa* Isolated from Different Ecological Niches: A Case Study in the Development of Multidrug Resistance in Environmental Hotspots

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Abstract: *Pseudomonas aeruginosa* is an opportunistic pathogen displaying high intrinsic antimicrobial resistance and the ability to thrive in different ecological environments. In this study, the ability of *P. aeruginosa* to develop simultaneous resistance to multiple antibiotics and disinfectants in different natural niches were investigated using strains collected from clinical samples, veterinary samples, and wastewater. The correlation between biocide and antimicrobial resistance was determined by employing principal component analysis. Molecular mechanisms linking biocide and antimicrobial resistance were interrogated by determining gene expression using RT-qPCR and identifying a potential genetic determinant for co- and cross-resistance using whole-genome sequencing. A subpopulation of *P. aeruginosa* isolates belonging to three sequence types was resistant against the common preservative benzalkonium chloride and showed cross-resistance to fluoroquinolones, cephalosporins, aminoglycosides, and multidrug resistance. Of these, the epidemiological high-risk ST235 clone was the most abundant. The overexpression of the MexAB-OprM drug efflux pump resulting from amino acid mutations in regulators MexR, NalC, or NalD was the major contributing factor for cross-resistance that could be reversed by an efflux pump inhibitor. This is the first comparison of antibiotic-biocide cross-resistance in samples isolated from different ecological niches and serves as a confirmation of laboratory-based studies on biocide adapted isolates. The isolates from wastewater had a higher incidence of multidrug resistance and biocide-antibiotic cross-resistance than those from clinical and veterinary settings.

Keywords: antimicrobial resistance; biocide resistance; efflux pump; *Pseudomonas aeruginosa*; cross-resistance; resistance development; selective pressure

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1. Introduction

Pseudomonas aeruginosa is a ubiquitous bacterium of environmental origin that is responsible for difficult-to-treat nosocomial infections such as wound, urinary tract, and other infections [1–4]. It is also a major cause of respiratory infections and the main cause of mortality in cystic fibrosis patients [5]. *P. aeruginosa* is a highly adaptive and robust organism that can thrive in a wide range of environmental niches owing to its large and dynamic genome that provides extraordinary metabolic versatility and genetic plasticity [6]. *P. aeruginosa* strains display high intrinsic antimicrobial resistance as well as the ability to tolerate a variety of biocides such as antiseptics, disinfectants, and preservatives [6–8]. Recently, *P. aeruginosa* has been classified by the World Health Organization as an organism of the most critical priority in need of new drug development. This is due to the global emergence of multidrug-resistant (MDR) high-risk clones, which are resistant to almost all available antipseudomonal drugs in clinical settings [9,10].

Among the antipseudomonal drugs, fluoroquinolones (FQs) are among the first line of antimicrobials used to treat *P. aeruginosa* infections [11,12]. FQs are potent, broad-spectrum antimicrobial agents with excellent bioavailability [8] and are characterized by a high degree of persistence in the environment [13]. Several studies have investigated the association between increased consumption of FQs and FQ resistance among *P. aeruginosa* isolates [14,15], while in vitro experiments have also demonstrated biocide exposure as a driver of antimicrobial resistance [16,17]. Biocides, which include disinfectants, antiseptics, and preservatives, are widely used as a part of infection prevention and control for various applications in both medical and household products [18]. For example, benzalkonium chloride (BKC) is widely used for the disinfection of surfaces and instruments [19] and also as a preservative in non-sterile formulations [20]. Triclosan is used in hand soaps and a large variety of everyday products such as toothpaste, mouthwash, deodorant, shower gel, hand lotion, hand cream, and hand sanitizer [21]. Chlorhexidine is widely used in healthcare for preoperative decontamination and in oral health antiseptics [7]. The use of such biocides unavoidably results in the generation and release of long-lasting residues [19]. The exposure of bacteria to such biocides at subinhibitory concentrations altered the microbial community composition [16,17,22,23] and increased tolerance to biocides as well as resistance to the most critically important antimicrobials used for the treatment of resistant Gram-negative bacteria such as FQs and beta-lactams [16,17,24–26]. Such co-selection may result from a number of antimicrobial-resistant (AMR) mechanisms, including drug extrusion by multidrug efflux pumps and mutation of the drug target encoding genes [16,17].

In *P. aeruginosa*, intrinsic resistance is largely attributed to the expression of multidrug efflux pumps belonging to the Resistance-Nodulation-Division (RND) family [27–29]. These pumps are chromosomally encoded membrane proteins forming tripartite complexes composed of an inner membrane transporter protein, a periplasmic adapter protein, and an outer-membrane channel protein [30,31]. Together, these proteins form a highly effective efflux pump able to expel a wide spectrum of structurally unrelated antimicrobial agents from the cell [28]. Genomic analysis has identified structural genes for at least 12 RND type efflux systems, of which four pumps MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM have been confirmed to play a role in MDR [32]. Of these, the MexAB-OprM is the most promiscuous and constitutively expressed multidrug efflux pump in *P. aeruginosa* and confers basal resistance to a wide range of antimicrobials [28,33]. Exposure to certain substrates or stresses leads to overexpression of the MexAB-OprM efflux pump (predominantly through mutations in regulatory genes such as *mexR*, *nalC*, or *nalD*) [34]. As a result of the promiscuous substrate profile of this efflux pump, the reduced accumulation of many different compounds including antibiotics, dyes, detergents, and disinfectants will be observed, leading to MDR *P. aeruginosa* [33,35].

In combination with the overexpression of multidrug efflux pumps, repertoires of chromosomal mutations in the quinolone-resistance-determining regions of DNA gyrase and topoisomerase IV-encoding genes *gyrA*, *gyrB*, *parC*, and *parE* contribute for high-level FQ resistance among *P. aeruginosa* isolates [36–38]. An in vitro experimental study demonstrated that mutations in the *gyrA* gene combined

with overexpression of the MexAB-OprM efflux pump are associated with a high-level resistance to FQ [>16 times increase in FQ minimum inhibitory concentration (MIC) compared to wild type] [24,38].

The highly adaptive and persistent nature of *P. aeruginosa* [6] coupled with the frequent use of biocides and antimicrobials in healthcare settings exert a further selective pressure on *P. aeruginosa* to acquire antimicrobial resistance. In particular, wastewater from healthcare sites has been defined as a "hot spot" for the acquisition of antimicrobial resistant determinants [39], as it serves as a reservoir for both antimicrobial residues and bacteria, presenting the perfect environment for the co-selection and development of antimicrobial resistance [19,24,40,41]. Although *P. aeruginosa* is known to be intrinsically resistant to biocides such as triclosan [42], epidemiologic cut-off values (ECOFF) for biocides are not currently available through the European Committee on Antimicrobial Susceptibility Testing (EUCAST) or Clinical and Laboratory Standards Institute (CLSI), making the determination of biocide resistance difficult.

While the link between increased biocide tolerance and antimicrobial cross-resistance has been experimentally documented in *P. aeruginosa* [17,24,43], these laboratory-based findings have not yet been observed in diverse natural niches (clinical, veterinary, and wastewater). This provides evidence of a relationship between biocide use and cross-resistance to clinically used antibiotics.

The aim of this study was to assess the correlation between biocide and antimicrobial resistance on a diverse sample set of *P. aeruginosa* isolates collected from clinical, veterinary, and wastewater sources. Biochemical and in-depth bioinformatic analysis of whole genome sequencing data was employed to investigate the molecular mechanism linking increased biocide resistance to antimicrobial resistance.

2. Materials and Methods

2.1. Source and Identification of P. aeruginosa Isolates

A total of 147 *P. aeruginosa* isolates were collected: 89 from humans, 20 from companion animals, and 38 from wastewater sources. Clinical isolates were collected from 2012 to 2017 at two hospitals in South Australia, from the USA and from the Netherlands from various clinical infections [44]. Animal isolates were collected in 2013 (Australian collection) from canine otitis, and wastewater isolates were collected from healthcare-generated wastewater in South Australia. Each isolate was sub-cultured on Columbia agar plates and identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) (Bruker Daltonik GmbH, Bremen, Germany). All confirmed isolates were stored at −80 °C for further microbiological and molecular studies.

2.2. Antimicrobial Susceptibility Testing

The minimum inhibitory concentrations (MICs) for antimicrobials were determined by the broth microdilution technique as described by the EUCAST guidelines [45] and adapted for biocides. Antimicrobial susceptibility was determined according to the 2020 EUCAST (http://www.eucast.org/) ECOFF value. The following classes of antimicrobials and biocides were used: Cephalosporins: ceftazidime, cefepime; Aminoglycosides: gentamicin, tobramycin; Fluoroquinolones: ciprofloxacin, levofloxacin; Carbapenems: imipenem, meropenem; Polymyxins: colistin; Biocides: chlorhexidine digluconate, triclosan, and benzalkonium chloride. Isolates displaying resistance to three or more antimicrobial classes were considered to be MDR [46]. *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were included in each experiment as control strains.

Establishing Biocide Epidemiologic Cut-off Values

The MICs of biocides were determined in triplicate using the broth microdilution technique. ECOFF (with 95% cut-off) values were calculated using the ECOFF finder XL 2010 program (https://clsi.org/meetings/microbiology/ecoffinder/).

2.3. Efflux Pump Inhibition Using a Checkerboard Assay

MICs of biocides and antimicrobials in the presence of the efflux pump inhibitor, phenylalanine arginine β-naphthylamide (PAβN) (Sigma-Aldrich, St. Louis, Missouri, USA) were determined using a checkerboard assay following the steps outlined in a previous study [47]. Only strains that were resistant to FQs (MIC of ciprofloxacin ≥ 1 mg/L and levofloxacin ≥ 2 mg/L) as well as benzalkonium chloride (MIC ≥ 128 mg/L) and triclosan (MIC ≥ 512 mg/L) were subjected to the checkerboard assay in the presence of efflux pump inhibitors. To address the possibility that a high concentration of PAβN permeabilizes the outer membrane of the cell, a nitrocefin hydrolysis assay was performed as described previously [31,48,49]. In brief, a β-lactamase producing *P. aeruginosa* strain (isolate PA0536 in this study) was treated with PAβN (4 to 128 mg/L) in the presence of nitrocefin (a chromogenic β-lactam) at a final concentration of 32 mg/L. If PAβN causes permeabilization of the outer membrane, nitrocefin will be able to traverse the outer membrane more easily and be exposed to beta-lactamase. This will result in an increased rate of nitrocefin hydrolysis that can be observed as a color change from yellow (≈380 nm) to red (≈490 nm). Polymyxin B, a known outer membrane permeabilizer, was used as a positive control at a final concentration of 128 mg/L. In order to determine the effect of other efflux pumps (not inhibited by PAβN) on the MIC of triclosan, we determined the MIC of triclosan in the presence of carbonyl cyanide-chlorophenylhydrazone (CCCP), which is a proton motive force inhibitor at 12.5 µM using the broth dilution method [50].

2.4. Gene Expression Analysis by Reverse-Transcription Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

Reverse-transcription quantitative real-time polymerase chain reaction (RT-qPCR) was used to quantify the transcription level of *mexA*, encoding for the periplasmic adapter protein of the MexAB–OprM drug efflux pump. Isolates shown to be resistant to FQ (MIC of ciprofloxacin ≥ 8 mg/L) and BKC (MIC ≥ 128 mg/L) and showing a ≥ 4-fold reduction in the MIC for both antibacterial agents in the presence of PAβN were selected for RT-qPCR analysis. Overnight cultures of *P. aeruginosa* were diluted (1:100) and grown at 37 °C in a cation adjusted Muller Hinton broth, up to the mid-log phase (A_{660} of 0.5) followed by the addition of two times the MIC of ciprofloxacin for 30 min (shock). Total RNA was extracted using a combination of Trizol reagent (Ambion Thermo Fisher Scientific, Carlsbad, CA, USA) and the MN NucleoSpin®RNA (Macherey-Nagel GmbH and Co.KG, Duren Germany) kit following the manufacturer's instructions. Briefly, 1 mL of cells were pelleted and resuspended in Trizol and chloroform, and further purified using the NucleoSpin®RNA kit. To remove DNA to a completely undetectable level, RNase-free rDNase digestion and further RNA clean-up was performed using the same kit. RNA purity and concentration were determined using a DeNovix DS-11 + Spectrophotometer (DeNovix Inc., Wilmington, DE, USA) and samples stored at −80 °C.

RT-qPCR was performed using a magnetic induction cycler instrument (AdeLab Scientific, Adelaide, Australia) with a KAPA SYBR® FAST One-Step RT-qPCR master mix (2×) kit (Sigma-Aldrich, Sigma-Aldrich, St. Louis, Missouri, USA). A housekeeping gene, *rspl*, was used to normalize the transcriptional levels of target genes, which were further calibrated against the *P. aeruginosa* PAO1 control strain. The primer sequences for the target gene (*mexA*) are: *mexA_F* 5^1-AGACGGTGACCCTGAATACC-3^1; *mexA_R* 5^1-GTCGGCCTCGTAGGTGG-3^1; *rspl_F* 5^1-CCAACGGTTTCGAGGTTTC-3^1; *rspl_R* 5^1-ACCCTGCTTACGGTCTTTGA-3^1. Each PCR experiment was carried out in triplicate with the following cycling parameters; a reverse transcription step at 42 °C for 10 min, and reverse transcription inactivation at 95 °C for 3 min, followed by 40 cycles of PCR at 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 15 s for denaturation, annealing and extension, respectively. A no-template control (NTC) and no reverse-transcriptase control (NRT) were included in each experiment. The relative expression of *mexA* was analyzed using the $2^{\Delta\Delta cq}$ method and normalized against the expression of the PAO1 *rspl* gene [51].

2.5. DNA Extraction and Whole Genome Sequencing (WGS)

Genomic DNA was extracted using the MN NucleoSpin®Microbial DNA (Machery-Nagel GmbH and Co.KG, Duren, Germany) kit following the manufacturer's instructions. The quantification of extracted genomic DNA was determined using a DeNovix DS-11 + Spectrophotometer. WGS was conducted at a high-throughput sequencing facility at SA Pathology in South Australia. Sequencing libraries were prepared using the Nextera XT DNA library preparation kit (Illumina Inc., San Diego, Ca, USA), with modifications of the kit's protocol. Briefly, genomic DNA was fragmented, followed by the amplification of Nextera XT indices (Illumina Inc., San Diego, CA, USA) to the DNA fragments using a low-cycle PCR reaction. The amplicon library was then purified, and normalised manually. Whole-genome sequencing (WGS) was performed on the Illumina NextSeq 550 platform with NextSeq 500/550 Mid-Output kit v2.5 (300 cycles) (Illumina Inc., San Diego, Ca, USA).

2.6. WGS Assembly, Annotation and Analysis

Raw paired-end sequencing reads were assembled and annotated using the TORMES pipeline v.1.1 [52]. Multi-Locus Sequence Type (MLST) assignment was carried out using the mlst software (T. Seemann, https://github.com/tseemann/mlst) and the PubMLST database [53]. A pangenome comparison of all isolates was performed using roary2svg (T. Seemann, https://github.com/sanger-pathogens/Roary/blob/master/contrib/roary2svg/roary2svg.pl). Draft genomes were screened for antimicrobial resistance genes using ResFinder [54], Antibiotic Resistance Gene-ANNOTation (ARG-ANNOT) [55], and the comprehensive antibiotic resistance database (CARD) [56]. Genes conferring biocide resistance were identified using the BacMet database (http://bacmet.biomedicine.gu.se) [57]. The *P. aeruginosa* PAO1 genome was used as a reference strain (accession number NC_002516.2; GI 110645304).

Antimicrobial resistance proteins identified by CARD to be carrying a mutation were used in a protein Basic Local Alignment Search Tool (BLASTp) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to compare regions of similarity between sequences. Protein sequences identified by BLASTp were subsequently used to generate a multi-sequence protein alignment using CLUSTAL O v.1.2.4 [58] to determine the prevalence of the mutation in other proteins.

2.7. Genome Accession Numbers

The draft whole genome sequences for 15 *P. aeruginosa* isolates has been deposited at GenBank, the accession number of each isolate were as follows: JACVNM000000000 (PA0115), JACVNN000000000 (PA0404), JACVNO000000000 (PA0461), JACVNP000000000 (PA0471), JACVNQ000000000 (PA0507), JACVNR000000000 (PA0508), JACVNS000000000 (PA0532), JACVNT000000000 (PA0536), JACVNU000000000 (PA0545), JACVNV000000000 (PA0550), JACVNW000000000 (PA0555), JACVNX000000000 (PA0570), JACVNY000000000 (PA0571), JACVNZ000000000 (PA0585), JACVOA000000000 (CLN_26).

2.8. Statistical Analysis

Principal component analysis (PCA) was used to cluster isolates based on their antimicrobial resistance patterns, and correlation matrix was used to determine the significant association between biocide and antimicrobial resistance using RStudio, version 3.6.1 [59]. Significant reversion to susceptible MIC in the presence of PAβN was analyzed using the Wilcoxon signed-rank test using IBM SPSS statistical software version 26.0 (SPSS, Armonk, NY, USA). The statistical analysis of *mexA* expression was performed using student t-test and presented in mean ± standard deviation. A p-value of ≤ 0.05 was considered significant.

3. Results

3.1. P. aeruginosa Isolates from Wastewater Display a High Prevalence for Multidrug Resistance in Contrast to Clinical and Veterinary Isolates

A culture collection of 147 *P. aeruginosa* isolates was assembled from clinical samples, veterinary samples, and wastewater, and their susceptibility against five different classes of antimicrobials was determined. The highest frequency of resistance was shown to levofloxacin (36.7%), followed by ciprofloxacin (32.7%), ceftazidime (28.6%), cefepime (27.9%), imipenem (20.4%), gentamicin (20.4%), meropenem (16.3%), and tobramycin (12.2%) (Figure 1 and Table S1). None of the isolates were resistant to colistin. Twenty-eight (19%) isolates were resistant to at least three classes of antimicrobials and were defined as MDR. Of these, 53.6% originated from wastewater.

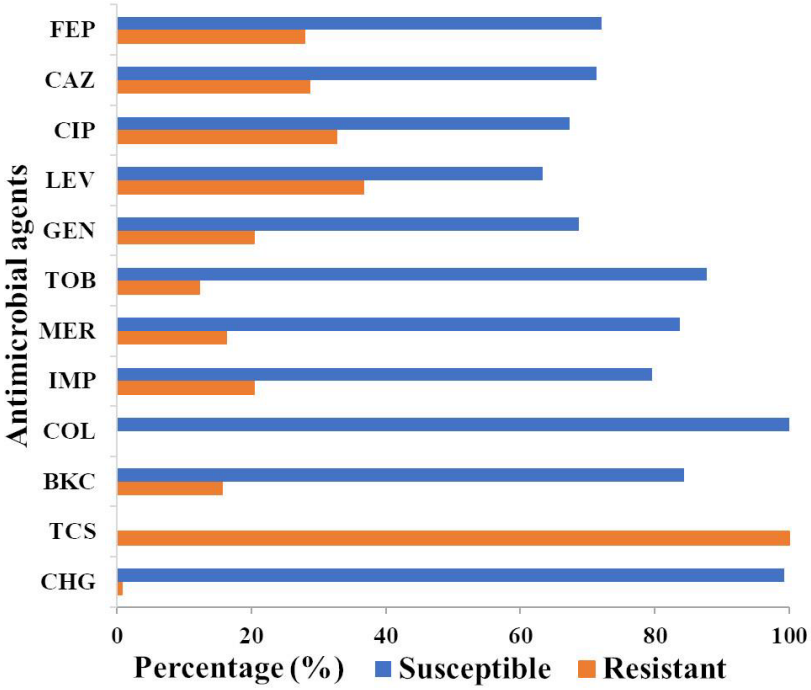

Figure 1. Proportions of antimicrobial susceptibility versus resistance in *P. aeruginosa*. Antimicrobial and biocide susceptibility against 147 *P. aeruginosa* isolates were based on the 2020, European Committee on Antimicrobial Susceptibility Testing (EUCAST) epidemiologic cut-off values (ECOFF) value. The ECOFF values for biocides were as determined in this study (Figure S1), since no ECOFF values were available for biocides against *P. aeruginosa*. The percentage of susceptible and resistant isolates was indicated in orange and blue, respectively. All 147 isolates of *P. aeruginosa* were intrinsically resistant to triclosan. FEP, cefepime; CAZ, ceftazidime; CIP, ciprofloxacin; LEV, levofloxacin; GEN, gentamicin; TOB, tobramycin; MER, meropenem; IMP, imipenem; COL, colistin; BKC, benzalkonium chloride; TCS, triclosan, CHG chlorhexidine digluconate.

3.2. P. aeruginosa Has a High Resistance against Common Biocides Used in Clinical Settings

Since antimicrobial resistance is often associated with an increased resistance to biocides, the resistance of *P. aeruginosa* against common biocides of clinical importance was investigated. There are currently no ECOFF values for biocides against *P. aeruginosa*; hence, the susceptibility of the 147 *P. aeruginosa* isolates against the three biocides, BKC, triclosan, and chlorhexidine digluconate, was established to determine the MIC distribution and to establish preliminary ECOFF values as a reference point for measuring resistance (non-wild type). Interestingly, almost all *P. aeruginosa* isolates were intrinsically resistant to triclosan with an MIC of > 1024 mg/L, only three clinical isolates showed a MIC value ranging from 64 to 256 mg/L (Figure 1, Table S1). The MIC data for chlorhexidine digluconate

and BKC exhibit normal distributions, ranging between three and five dilution steps and justified the criteria set by EUCAST for establishing ECOFF values with ECOFF values of 32 and 128 mg/L, respectively (Figure S2). Only one clinical strain displayed resistance to chlorhexidine digluconate, while twenty-three (15.6%) of the isolates had shown an MIC above the wild-type ECOFF value of 128 mg/L to BKC and hence were deemed BKC resistant.

3.3. Phenotypic Correlation between Biocide and Antimicrobial Resistance in P. aeruginosa Isolates

In order to establish if any relationship exists between antimicrobial and biocide resistance, a PCA was performed. Analyses of the MIC values using PCA revealed three clusters of antimicrobial resistance in *P. aeruginosa* isolates (Figure 2A). The first cluster showed isolates' resistance to BKC, FQs, and cephalosporins. The second cluster includes isolates resistant to aminoglycosides and carbapenems, while the third cluster exhibits isolates susceptible to colistin and chlorohexidine digluconate (Figure 2A). The MIC values of triclosan were not included in the PCA analysis due to the intrinsic resistance of *P. aeruginosa* against triclosan (Table S1). To further investigate the dependence between multiple variables at the same time, a correlation matrix was used. The result showed a significant correlation between BKC resistance and the antimicrobial classes FQs, cephalosporins and aminoglycosides (Figure 2B). Since a large number of BKC-tolerant isolates were also resistant to FQs as shown in Figure 2A, further investigation into the underlying mechanisms for cross-resistance between BKC tolerant and ciprofloxacin-resistant subpopulations of *P. aeruginosa* isolates was carried out.

3.4. Efflux Pump Inhibition Reveals RND Pump Mediated Biocide and Antimicrobial Resistance

In order to investigate the putative role of drug efflux pumps in the observed antimicrobial and biocide resistance, the effect of the efflux pump inhibitor PAβN on BKC, triclosan, and ciprofloxacin resistance was determined. All BKC-tolerant and 37.5% of the ciprofloxacin-resistant *P. aeruginosa* isolates were reversed back to sensitive levels when tested in the presence of PAβN (example Figure 3, Table S2). However, PAβN did not modify the MIC of triclosan for any of the isolates tested (Table S2). To explore other potential efflux pumps that may be responsible for triclosan intrinsic resistance but not inhibited by PAβN, we used CCCP. Nonetheless, the reversion of triclosan resistance was not shown upon the addition of CCCP, either (Table S2). To verify that the observed reduction in the MIC of BCK and ciprofloxacin in the presence of PAβN at 32 mg/L was as a direct result of inhibition of the RND-type drug efflux pumps and not due to any non-specific outer membrane permeability effects, the integrity of the outer membrane was determined in the presence of PAβN at 32 mg/L using the nitrocefin hydrolysis rate as an indicator of outer membrane integrity [47,49]. The results clearly indicate no increase in the nitrocefin hydrolysis rate in the presence of PAβN; hence, the reduction in MIC values observed with PAβN was solely due to the inhibition of active efflux (Figure S2).

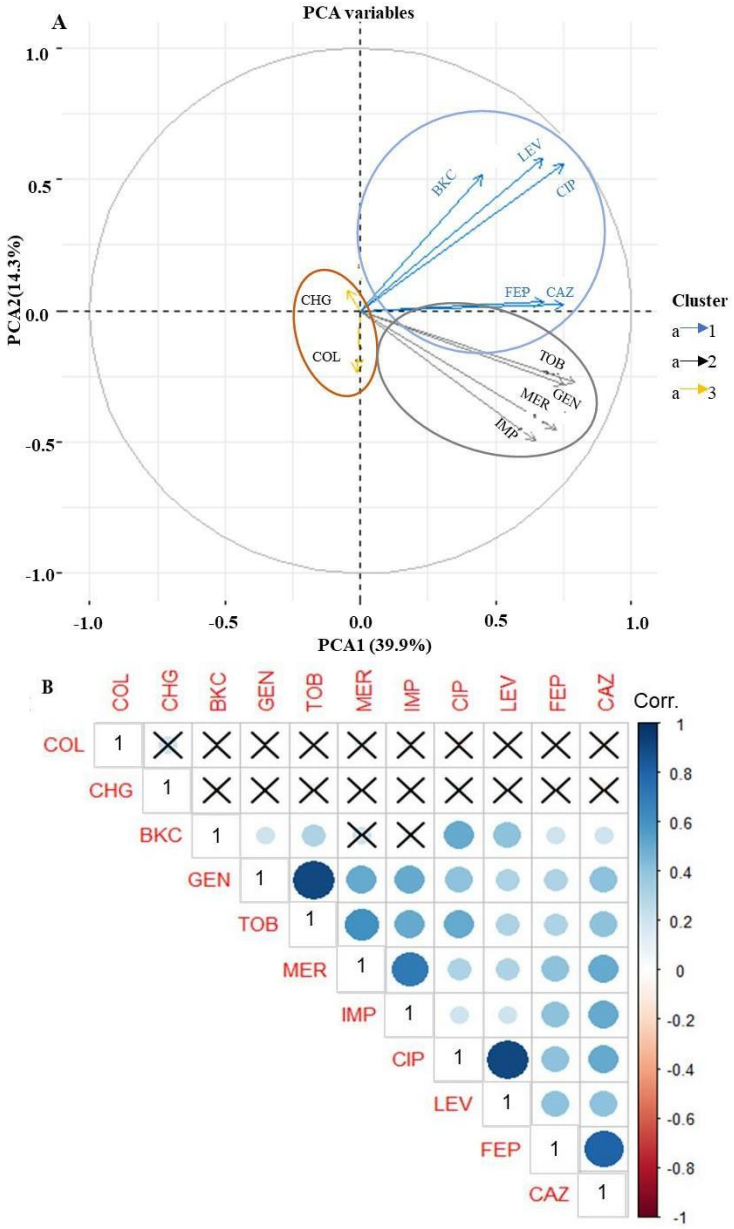

Figure 2. BKC resistance correlate with antimicrobial resistance. Principal component analysis (PCA) and Pearson correlation coefficient of 147 *P. aeruginosa* isolates (variables) against five classes of antimicrobials and two biocides based on minimum inhibitory concentration (MIC) value (scores). Colors are used to differentiate isolates with similar clusters of antimicrobial susceptibility patterns

(**A**). The correlation matrix indicates a significant correlation between BKC and antimicrobial classes FQs, cephalosporins, and aminoglycosides. The intensity of the blue circles indicates the correlation coefficient value, and the sizes indicate the significance of correlation ($p < 0.05$). The bar to the right of the figure indicates the color coding of the correlation value from +1 (strong positive correlation) to −1 (strong negative correlation). Coordinates marked with "×" indicate no significant correlation (p value > 0.05) (**B**). CAZ, ceftazidime; FEP, cefepime; LEV, levofloxacin; CIP, ciprofloxacin; IMP, imipenem; MER, meropenem; TOB, tobramycin; GEN, gentamicin; COL, colistin; BKC, benzalkonium chloride; CHG chlorhexidine.

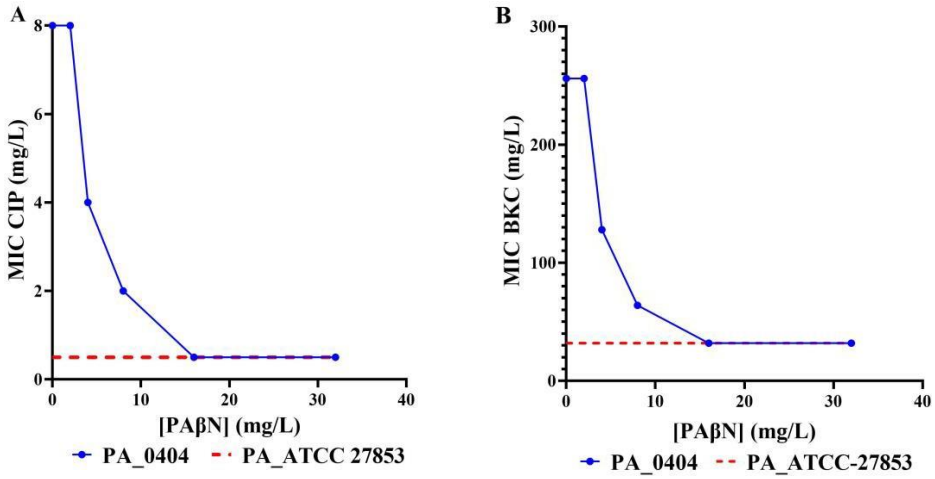

Figure 3. Reversal of resistance in the presence of an efflux pump inhibitor. Effect of efflux pump inhibitor, (PaβN) in the CIP-resistant and BKC-resistant PA0404 isolate in comparison to a susceptible strain. The MIC values for CIP (**A**) and BKC (**B**) of the CIP and BKC-resistant PA0404 isolate were determined in the presence of increasing concentrations of the efflux pump inhibitor PAβN (blue line). For reference, the MIC for the wild-type reference strain—*P. aeruginosa* ATCC—27853 are indicated, too (red broken line).

3.5. RT-qPCR Revealed MexAB-OprM efflux Pump Overexpression

Due to the effect of the efflux pump inhibitor PAβN on ciprofloxacin and BKC resistance, it was hypothesized that the cross-resistance between these compounds could be mediated by the overexpression of multidrug efflux pumps. MexAB-OprM is the most promiscuous and constitutively expressed efflux pump that has a significant role in natural resistance of *P. aeruginosa*. To verify the role of MexAB-OprM in cross-resistance, 15 isolates showing an MIC reduction by at least 4-fold in the presence of PAβN were selected for RT-qPCR. Results revealed an overexpression of the MexA pump, showing a 1.896- to 5.911-fold (mean 3.1) difference between the 15 isolates examined and the wild-type PAO1 strain (Table 1, Figure 4). Almost all MexAB-OprM overexpressing isolates are resistant to BKC and showed cross-resistance to FQs resistance, cephalosporins resistance, and MDR subpopulations. The highest level of overexpression (≈6 fold) of MexA was observed for the clinical isolate. Since this clinical isolate was isolated from cystic fibrosis patients, the high-level overexpression is consistent with the role of MexAB-OprM in invasion [60] in addition to its role in antimicrobial resistance.

3.6. WGS Analysis Reveals Multiple Mutations in the Regulators of the mexab-oprM Efflux Pump

WGS analysis was carried out to confirm the molecular mechanism for the link between biocide and FQ resistance in the same 15 *P. aeruginosa* isolates as mentioned above. Antimicrobial resistance genes were identified using three databases with the CARD identifying not only AMR genes and proteins but also AMR protein variants and their mutations [56]. Further interrogation of the WGS data with blast analysis and multiple sequence alignment revealed multiple amino acid mutations in the regulatory genes of the *mexAB-oprM* efflux pump (Table 1, Figure 5), which may lead to the observed increased expression of this efflux pump (Figure 4) and subsequent high-level resistance to BKC and FQs. Of these, three non-synonymous mutations were found in NalC proteins, including G71E, S209R, and E153Q, and one mutation (V126E) was found in the MexR regulator. In addition to these mutations, isolate PA0570 had a 13 amino acid truncation at the beginning of the NalC regulator protein. Isolate PA0404, with a fairly high overexpression fold of 4.7, contained an additional T11N mutation in the NalD repressor protein and displayed a novel combination of mutations in the MexR, NalC, and NalD proteins (Table 1).

Table 1. Phenotypic and genotypic characteristics of the 15 MexAB-OprM efflux pump overexpressing *P. aeruginosa* isolates, determined by using conventional MICs method, RT-qPCR, and WGS.

Strains	ST	Antimicrobial Susceptibility									RP	MexAB-OprM	Fluoroquinolone Resistance Determinants				Mutation in MexAB-OprM Efflux Pump Regulators				Triclosan
		FEP	CAZ	CIP	LEV	GEN	TOB	MER	IMP	COL			GyrA	GyrB	ParC	ParE	CrpP	MexR	NalC	NalD	FabV
PAO1		S	S	S	S	S	S	S	S	S	S	1									
PA0115	235	R	R	R	R	R	S	S	S	S	MDR	2.622	T83I	–	S87L	D533E	–	V126E	S209R, G71E, E153Q	–	P36L
PA0404	235	R	R	R	R	R	R	S	R	S	MDR	4.705	T83I	–	S87L	D533E	–	V126E	S209R, G71E, E153Q	T11N	P36L
PA0461	235	R	R	R	R	R	R	R	R	S	MDR	1.986	T83I	–	S87L	D533E	–	V126E	S209R, G71E, E153Q	–	P36L
PA0471	235	R	R	R	R	S	S	R	R	S	MDR	2.462	T83I	–	S87L	D533E	–	V126E	S209R, G71E, E153Q	–	P36L
PA0507	235	R	R	R	R	R	S	S	S	S	MDR	1.896	T83I	–	S87L	D533E	–	V126E	S209R, G71E, E153Q	–	P36L
PA0508	235	R	R	R	R	R	R	S	S	S	MDR	3.778	T83I	–	S87L	D533E	–	V126E	S209R, G71E, E153Q	–	P36L
PA0532	235	R	R	R	R	R	R	R	R	S	MDR	2.097	T83I	–	S87L	D533E	–	V126E	S209R, G71E	–	P36L
PA0536	815	R	R	R	R	R	R	R	R	S	MDR	2.707	D87N	–	V297I	D533E	+	V126E	S209R, G71E	–	P36L
PA0545	815	R	R	R	R	R	R	R	R	S	MDR	2.610	D87N	–	V297I	D533E	+	V126E	S209R, G71E	–	P36L
PA0550	235	R	R	R	R	R	S	S	S	S	MDR	2.882	T83I	–	S87L	D533E	–	V126E	S209R, G71E, E153Q	–	P36L
PA0555	235	R	R	R	R	S	S	S	S	S	NMDR	1.969	T83I	–	S87L	D533E	–	V126E	S209R, G71E, E153Q	–	P36L
PA0570	235	R	R	R	R	R	S	S	S	S	MDR	2.486	T83I	–	S87L, TR*	D533E	–	V126E	S209R, G71E, E153Q, TR***	–	P36L
PA0571	235	R	R	R	R	R	S	S	S	S	MDR	3.427	T83I	–	S87L	D533EE569-**	–	V126E	S209R, G71E, E153Q	–	P36L
PA0585	235	R	R	R	R	R	S	S	S	S	MDR	1.996	T83I	–	S87L	D533E	–	V126E	S209R, G71E, E153Q	–	P36L
CLN_26	274	R	R	R	R	S	S	S	S	S	NMDR	5.911	T83I	E468D, H148N	–	P438S, L501F	–	–	S209R, G71E	–	P260T

CLN: clinical strains; PA: wastewater strains; PAO1: reference strain; ST, sequence type; RP: resistance profile; S: susceptible; NMDR: non-multidrug resistant; MDR: multidrug resistant; E569-** one amino acid deletion at 569 amino acid position of ParE; TR*: 33 amino acid truncation at the C-terminal; TR*** 13 amino acid truncation at the N-terminal; –: no mutation; V: valine; P: proline; T: threonine; D: aspartate; L: leucine; S: serine; E: glutamate; Q: glutamine; I: isoleucine; R: arginine G: glycine; F: phenylalanine; WGS: whole genome sequencing; MIC: minimum inhibitory concentration; RT-qPCR: reverse-transcription quantitative real-time polymerase chain reaction; CAZ: ceftazidime; CIP: ciprofloxacin; COL: colistin; GEN: gentamicin; FEP: cefepime; IMP: imipenem; LEV: levofloxacin; MER: meropenem; TOB: tobramycin.

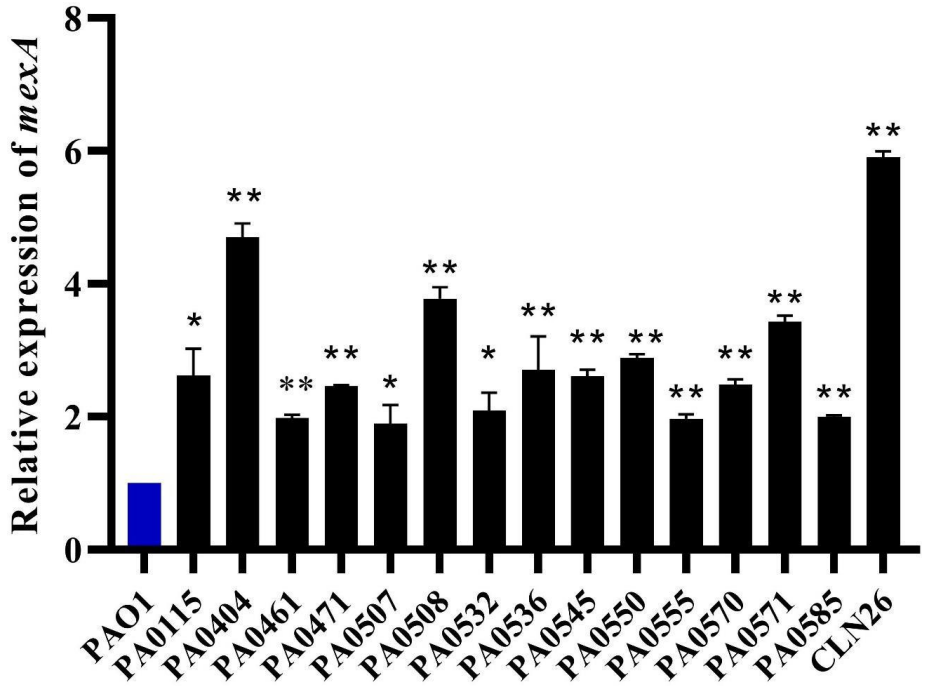

Figure 4. *MexA* is overexpressed in BKC-tolerant and ciprofloxacin-resistant isolates. The expression levels of the *mexAB-oprM* operon, as assessed by RT-qPCR of the *mexA* gene, reveal the overexpression of *mexAB-oprM* in BKC-tolerant and CIP-resistant isolates (black bars) as compared to the wild-type PAO1 strain (blue bar) (set at a value of 1, by definition). The results are presented in mean ± standard deviation. Statistical analysis was performed using Student t-test and statistical significance was represented with asterisks (*) as shown in the figure (* $p < 0.01$; ** $p < 0.001$).

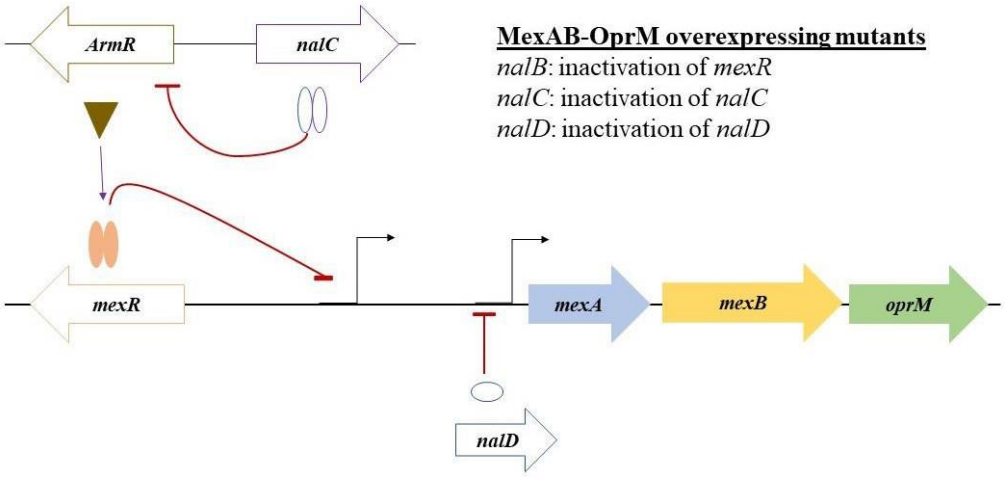

Figure 5. Schematic representation of the regulator genes involved in the repression of MexAB-OprM efflux pump in *P. aeruginosa*. The basal expression of the MexAB-OprM efflux pump in wild-type strains is controlled by a local repressor *mexR* (self-regulated) by binding as a homodimer to the distal promoter of the *mexAB-oprM*. A second repressor, NalD, directly binds as a monomer to the proximal promoter of the *mexAB-oprM* Operon. The third repressor, *nalC*, has an indirect effect on *mexAB-oprM* expression; it represses the expression of gene *armR*, the product of which acts as an anti-repressor of *mexR*. Mutations in these repressors result in a high-level expression of MexAB-OprM, rendering stable, acquired resistance. Mutations in these three repressors have been reported in *mexR (nalB mutation), nalD*, and *nalC* mutants, respectively (Table 1).

WGS analysis also revealed that two isolates, PA0508 and PA0532, with MIC values for BKC equal to the ECOFF value, lack the wild-type *mexEF-oprJ* MDR efflux system and its corresponding regulator genes, *mexT* and *mexS*. None of the isolates displayed any mutations in the amino acid sequence of the regulator genes of the *mexCD-oprN*, *mexEF-oprJ*, or *mexXY-oprM* efflux pump systems.

Collectively, the data confirm MexAB-OprM efflux pump overexpression and its role in the cross-resistance of BKC and FQ.

3.7. The Role of Other Resistance Gene Determinants in the Development of High-Level Resistance to FQs and Biocides

Given that 14 sequenced *P. aeruginosa* isolates displayed high levels of FQ resistance that could not be completely reversed by the efflux pump inhibitor PAβN (Table S2), we investigated the role of other resistance mechanisms using WGS. All 15 of these *P. aeruginosa* isolates harbored mutations in the DNA gyrase and/or topoisomerase IV proteins (GyrA/B and ParC/E). The main amino acid mutations noted were T83I and D87N in GyrA, S87L and V297I in ParC, and D533E in ParE. A 33 amino acid truncation of the ParC protein was also observed in one isolate (PA0571) as well as a one amino acid deletion in the ParE protein (PA0570). One isolate (CLN26) carried two mutations in the GyrB protein (E468D and H148N) and another carried two mutations in the ParE protein (P438S and L501F) in combination with a T83I mutation in GyrA (Table 1). Although showing no difference to the level of resistance, the ciprofloxacin-modifying *crpP* gene was also identified in two of the isolates (PA0536 and PA0545). A single amino acid mutation in the GyrA/B or ParC/E (conserved mutations, as observed in these and other *P. aeruginosa* isolates) is seldom sufficient to confer high-level FQ resistance. However, it is possible that the cumulative effect of these mutations could contribute to the high level of FQ resistance observed.

To screen the presence of resistance genes to BKC and triclosan, the BacMet database was used. All isolates that are resistant to triclosan were shown to carry the triclosan-resistant enoyl-acyl-carrier protein reductase *fabV* gene encoding for the FabV protein (Table 1).

3.8. The Pangenome Analysis Indicated an Evolutionary Divergence between mexAB-oprM Efflux Pump Overexpressing P. aeruginosa Isolates

To decipher the genetic relationships among MDR *P. aeruginosa* isolates overexpressing the *mexAB-oprM* efflux pump system, whole-genome MLST and pangenome analysis was conducted on 15 *P. aeruginosa* isolates. Three different MLSTs were identified in the study, twelve isolates of which were represented by a single ST, which is the known epidemiological high-risk clone ST235. All of the isolates belonging to ST235 were recovered from wastewater. The remaining *P. aeruginosa* isolates belonged to ST815 (n = 2), also isolated from wastewater and ST274 (n = 1) isolated from a clinical sample. To further confirm that the dominant ST235 isolates are not diffusion of the same clone as a result of antimicrobial selection pressure, a pangenome analysis was carried out, with the results showing an evolutionary divergence between ST235 isolates (Figure S3).

4. Discussion

Our study on a collection of *P. aeruginosa* isolated from diverse ecological niches revealed a relatively high proportion (19%) of MDR isolates. This number is much higher than what the Australian Group on Antimicrobial Resistance surveillance found in bacteremic patients (4.3%) [61] and higher than the European Union and European Economic Area surveillance population-weighted mean resistance (12.8%) [62]. The reason for the high proportion of MDR could be the inclusion of isolates from healthcare-generated wastewater which accounted for more than half of the MDR isolates in our study. Multiple factors could contribute to the increased antimicrobial resistance observed in isolates from healthcare wastewater, such as high antimicrobial and disinfectant use in healthcare settings, as well as patients on prolonged courses of antimicrobials who could harbor and shed resistant isolates [19,63].

Most of our isolates originating from wastewater environments demonstrated increased resistance to BKC and cross-resistance for FQ and MDR (55.5% of the total wastewater isolates). This fraction is significantly higher than that observed for isolates from other ecological niches. This difference could be attributed to the difference in the concentration and length of exposure to these agents in these two environments. In the clinical environment, biocides and antibiotics are used at inhibitory concentrations and are less likely to select tolerant clones [64], whereas residues exist at subinhibitory concentrations in the wastewater environment that could provide a selective pressure for the development of resistant subpopulations to thrive and predominate [65].

Studies on laboratory-adapted biocide insensitive *P. aeruginosa* indicated a correlation with antimicrobial resistance with efflux pump overexpression as the main mechanism of resistance [17,24,66–68] and reviewed in [43,69]. These observations have led to the hypothesis that there could be a correlation between biocide use and the development of antimicrobial resistance in clinical or environmental situations, although this has never been translated from laboratory-adapted strains to a confirmation in the natural environment. Our study is the first to provide evidence for the correlation between biocide and antibiotic cross-resistance driven by efflux pump overexpression on isolates from native niches that have "adapted" to their environment. It is also the first study of naturally developed cross-resistance that confirms findings previously only seen in laboratory-based studies. Our work also bestows credence to previous suggestions that healthcare-associated effluent could act as a hotspot for antimicrobial resistance development [39]. The reversal of resistance by the efflux pump inhibitor PAβN, direct measurement of efflux pump expression, and WGS confirmed mutational hyperexpression of the chromosomally encoded MexAB-OprM efflux pump as the main reason for the observed resistance and cross-resistance to antibiotics. The upregulation of MDR efflux pumps provides a fitness advantage that is central to the survival of an organism at low concentrations of a diverse range of xenobiotics such as is found in the wastewater environment [17,24,26,70].

Whole-genome analysis of MLSTs revealed an overrepresentation of a subpopulation of *P. aeruginosa* wastewater isolates belonging to ST235, which has been described as an MDR gene-enriched international high-risk clone isolated from wastewater [3], human, animal, and other niches [8]. Studies suggest that the global spread of ST235 has been potentially associated with the selective pressure of FQs [9]. Interestingly, we also identified two wastewater isolates that belong to ST815, which appear to be uncommon but are resistant to four major antipseudomonal drugs, including carbapenems. The only record of ST815 comes from a study in Portugal [71]. These isolates also carried the ciprofloxacin-modifying *crpP* resistance gene (50, 51), which has been shown to marginally increase MICs to FQs through ciprofloxacin phosphorylation [72]. The reduced susceptibility mediated by this enzyme is not to the level of clinical resistance; however, the presence of this enzyme facilitates the selection of mutants that generate quinolone resistance and promote treatment failure [36]. Only a single clinical isolate identified from a cystic fibrosis patient showing cross-resistance between BKC and FQs belongs to ST274. This ST is another epidemic high-risk clone circulating in European countries, mainly Spain, and Australia and has previously been isolated from both cystic and non-cystic fibrosis patients [73,74].

Finally, we found that every *P. aeruginosa* isolate in this study exhibits an intrinsic resistance to triclosan. Sequencing confirmed the presence of the *fabV* gene, coding for the triclosan-resistant enoyl-acyl-carrier protein in each of these isolates [42]. Before the US Food and Drug Administration banned the use of triclosan (www.fda.gov) in 2016, triclosan was extensively used in common household products such as antimicrobial soaps and other cleaning products. Given the propensity of triclosan to select for AMR [70], this move would help prevent the further development of triclosan and concomitant AMR. However, due to the acquisition of the *fabV* gene conferring high-level and stable triclosan resistance, the proportion of triclosan-resistant populations appears to be stable [42]. Biocides are also frequently used in mass-marketed household products, which could lead to the development of resistance and have severe consequences for their clinical usefulness [75,76]

Our study clearly shows that cross-resistance between BKC and FQ, as well as MDR, are driven by hyperexpression of the drug efflux pumps and occurs spontaneously in a natural environment. This should serve as a warning that the unlimited and uncontrolled use of biocides has repercussions as BKC plays a role in the development of resistance against our most useful classes of antimicrobials used to treat resistant Gram-negative infections. It also shows the power of efflux pumps as mechanisms of resistance that continue to evolve, ever expanding their capacity to protect bacterial cells against the very antimicrobials we use for treatment. As such, greater restrain in the use of biocides is needed to preserve the usefulness of these important chemicals and to extend the lifetime of currently used antimicrobials.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2607/8/11/1647/ s1, **Table S1**. Minimum inhibitory concentration distribution of antimicrobial agents against *P. aeruginosa* (n = 147) isolates collected from clinical, veterinary and wastewater, **Table S2.** Effects of PAβN on the MIC value of P. aeruginosa isolates resistant to an antibiotic (e.g., ciprofloxacin) and biocides: BKC and triclosan. **Figure S1**: ECOFF value determinations for chlorohexidine digluconate and BKC, **Figure S2**. Evaluating the outer membrane-permeabilizing activity of PAβN, **Figure S3**. Visualization of pangenome-analysis of 15

P. aeruginosa isolates.

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Appendix 2. Antimicrobial susceptibility profile of C. freundii isolated from hospital wastewater

C. freundii		Class	Cephalosporins		Fluoroquinolones		Aminoglycosides		Carbapenems	Polymyxin	Disinfectants			Heavy Metal
		Antimicrobial	FEP	CAZ	CIP	LEV	GEN	TOB	MER	COL	CHL	TRI	BNZ	$AgNO_3$
		EUCAST breakpoint	0.008-0.125	0.016-1	0.002-0.125	NA	0.125-2	0.25-2	0.016-0.25	0.0125-2	64	NA	32	64
Site	Isolate	Plated from												
Site 1	297	Terg0	>64	64	8	1	>64	8	<0.125	<0.125	32	>64	32	8
	322	TergC+	32	>128	16	4	128	16	<0.25	<0.0625	32	>64	16	4
	330	Terg0	64	64	4	1	>64	16	<0.125	<0.125	16	>64	32	>64
	331	Terg0	>64	>64	>64	4	64	16	<0.125	0.25	8	>64	32	>64
	339	TergC+	32	128	8	2	32	16	<0.25	<0.125	8	>64	NA	NA
	340	TergC+	>64	>64	4	1	>64	32	0.125	<0.125	NA	NA	NA	NA
	342	TergC+	>64	64	8	1	>64	32	0.25	<0.125	16	>64	16	16
	343	TergC+	>64	64	4	2	>64	32	0.25	0.25	16	>64	16	32
	344	TergC+	>64	64	8	2	>64	32	0.25	0.25	16	>64	32	16
	409	Ps0	16	32	4	1	16	4	0.25	<0.125	16	>64	32	4
	412	Ps0	8	>64	16	2	>64	16	<0.125	<0.125	32	>64	16	8
Site 2	428	BileV+	0.5	64	1	2	>64	64	<0.125	<0.125	16	>64	32	16
	443	BileV+	0.5	32	1	1	>64	32	<0.125	<0.125	8	>64	NA	16
	512	Terg0	0.5	>64	<0.125	<0.125	>64	2	<0.125	<0.125	0.125	>512	16	8
	515	TergC+	<0.25	32	1	4	128	1	<0.125	0.25	64	>64	NA	2
	516	TergC+	2	>128	2	<0.25	>128	16	<0.125	>0.0625	16	>512	8	2
	589	Terg0	<0.125	16	1	2	64	4	<0.125	<0.125	16	>64	16	4
	611	BileV+	0.5	8	64	32	32	32	<0.125	<0.125	4	>64	16	8
	668	Terg0	>64	>64	32	8	>64	64	NA	<0.125	16	>64	64	8
	677	Terg0	0.25	16	1	1	>64	16	0.25	NA	16	>64	16	8
Site 3	193	XLDC+	>128	>128	2	<0.25	16	NA	<0.125	NA	32	>64	NA	NA
	595	Terg0	32	32	32	0.5	64	16	<0.125	0.5	0.5	>512	32	>64
	596	Terg0	<0.25	16	0.5	<0.25	32	4	<0.125	<0.0625	16	>64	NA	1
Site R	834	Terg0	<0.25	0.5	<0.25	<0.25	0.5	<0.25	<0.125	<0.0625	2	>512	16	1
	843	Ps0	<0.125	64	0.25	1	>64	0.5	<0.125	<0.03125	32	>64	16	16

The minimum inhibitory concentrations were determined for C. freundii isolates isolated from wastewater from four different sites at one hospital: Site 1: Radiology/Oncology; Site 2: Residential Site 3: Theatre/Emergency/Outpatient; and Site 4: Reference site. Isolates were recovered from various selective media: Tergitol-7 without additional antibiotics Terg0; Tergitol-7 with 1mg/L of ciprofloxacin TergC; Pseudomonas agar without antibiotics Ps0; bile aesculin with 4mg/L of vancomycin BileV+ and XLD with 1mg/L of ciprofloxacin XLD C+. Eight different antibiotics: cefepime (FEP), ceftazidime (CAZ), ciprofloxacin (CIP), levofloxacin (LEV), gentamicin (GEN), tobramycin (TOB), meropenem (MER), colistin (COL) and 4 other antimicrobials: chlorhexidine (CHL), triclosan (TRI), benzalkonium chloride (BNZ) and silver nitrate ($AgNO_3$) were used. Results were compared to the epidemiological cut-off values, displayed in the table as mg/L, from the European Committee on Antimicrobial Susceptibility Testing [118]. Resistance/sensitivity is indicated as the following colours: resistance - salmon; sensitive- green, grey, unable to be determined. Isolates displaying resistance against more than one family of antibiotics were deemed multidrug resistant and are indicated in dark red.

Appendix 3. Antimicrobial susceptibility profile of E. cloacae isolated from hospital wastewater

E. cloacae		Class	Cephalosporins		Fluoroquinolones		Aminoglycosides		Carbapenems	Polymyxin	Disinfectants			Heavy Metal
		Antimicrobial	FEP	CAZ	CIP	LEV	GEN	TOB	MER	COL	CHL	TRI	BNZ	$AgNO_3$
		EUCAST breakpoint	0.032-0.125	0.016-1	0.002-0.125	NA	0.064-2	0.25-2	0.04-0.125	0.125-2	128	1	NA	32
Site	**Isolate**	**Plated from**												
Site 1	**388**	BileV+	2	>64	0.5	1	>64	32	0.5	0.125	16	<0.125	32	16
Site 2	**453**	BileV+	8	>64	1	0.5	64	16	<0.125	<0.125	64	<0.125	16	8
	454	BileV+	<0.125	2	0.25	0.5	64	16	4	1	16	8	16	16
	455	BileV+	1	4	2	4	>64	32	16	<0.125	32	1	8	8
	463	BileV+	>64	64	2	1	32	64	<0.125	<0.125	32	<0.125	16	8
	479	TergO	16	>128	4	4	>128	16	1	<0.125	2	1	16	16
	491	TergO	16	>128	2	4	32	8	2	<0.125		<0.125	16	16
	500	TergO	<0.125	1	<0.125	0.25	0.25	1	<0.125	<0.125	8	>64	16	32
	521	TergC+	8	>128	2	4	>128	NA	2	NA	2	0.5	NA	NA
	561	TergC+	>128	>128	16	8	64	NA	<0.125	NA	2	<0.125	NA	NA
	632	TergC+	8	>64	4	4	64	>64	0.25	<0.125	1	<0.125	8	8
Site 3	**669**	TergO	64	64	<0.125	0.5	32	16	1	NA	16	2	16	8
	692	BileV+	8	64	0.25	0.5	>64	16	32	<0.125	2	0.125	16	>64
	701	BileV+	>64	>64	2	8	0.25	0.25	0.25	<0.125	8	<0.125	16	4
	709	TergC+	2	4	4	NA	16	NA	NA	NA	NA	NA	NA	NA
	713	BileV+	1	4	4	2	>64	32	NA	NA	64	>64	16	>64
	724	TergC+	<0.25	4	2	1	8	0.25	4	<0.125	<1	<0.25	1	2
	745	BileV+	>64	>64	1	1	32	8	4	<0.125	1	<0.125	16	4
	746	BileV+	0.5	4	1	0.5	1	4	4	<0.125	0.5	<0.125	16	4
	768	TergO	>128	>128	<0.25	0.25	<0.25	0.25	<0.25	2	16	0.5	16	16
	770	TergO	128	>128	<0.25	<0.125	<0.25	0.25	<0.25	0.5	8	0.25	16	16
Site 4	**890**	TergO	<0.125	<0.125	<0.125	<0.125	0.25	0.25	<0.125	NA	1	<0.125	16	4
	891	TergO	<0.25	8	<0.25	<0.125	0.5	0.5	<0.25	NA	8	1	NA	NA
	945	TergO	<0.125	0.255	0.125	<0.125	0.25	0.25	1	<0.215	4	<0.125	<0.125	16
	946	TergO	<0.125	0.25	<0.125	<0.125	0.5	0.5	<0.125	<0.125	16	<0.125	4	16

The minimum inhibitory concentrations were determined for E. cloacae isolates isolated from wastewater from four different sites at one hospital: Site 1: Radiology/Oncology; Site 2: Residential Site 3: Theatre/Emergency/Outpatient; and Site 4: Reference site. Isolates were recovered from various selective media: Tergitol-7 without additional antibiotics Terg0; Tergitol-7 with 1mg/L of ciprofloxacin TergC; Pseudomonas agar without antibiotics Ps0; bile aesculin with 4mg/L of vancomycin BileV+ and XLD with 1mg/L of ciprofloxacin XLD C+. Eight different antibiotics: cefepime (FEP), ceftazidime (CAZ), ciprofloxacin (CIP), levofloxacin (LEV), gentamicin (GEN), tobramycin (TOB), meropenem (MER), colistin (COL) and 4 other antimicrobials: chlorhexidine (CHL), triclosan (TRI), benzalkonium chloride (BNZ) and silver nitrate ($AgNO_3$) were used. Results were compared to the epidemiological cut-off values, displayed in the table as mg/L, from the European Committee on Antimicrobial Susceptibility Testing [118]. Resistance/sensitivity is indicated as the following colours: resistance - salmon; sensitive- green, grey, unable to be determined. Isolates displaying resistance against more than one family of antibiotics were deemed multidrug resistant and are indicated in dark red.

Appendix 4. Antimicrobial susceptibility profile of E. coli isolated from hospital wastewater

E. coli		Class	Cephalosporins		Fluoroquinolones		Aminoglycosides		Carbapenems	Polymyxin	Disinfectants			Heavy Metal
		Antibiotic	FEP	CAZ	CIP	LEV	GEN	TOB	MER	COL	CHL	TRI	BNZ	$AgNO_3$
		EUCAST breakpoint	0.016-0.125	0.06-0.5	0.004-0.016	0.008-0.6	0.25-1	0.25-1	0.008-0.6	0.25-2	1	0.25	32	128
Site	Isolate	Plated from												
ATCC	25923	NA	0.125	0.25	0.008	0.015625	1	1	0.03125	0.25	0.5	0.25	8.0-16	2-16
Site 1	288	Terg0	64	>64	8	NA	16	16	NA	0.25	0.5	NA	8	64
	308	Terg0	64	32	0.5	1	>64	32	<0.125	<0.125	1	<0.125	16	8
	378	Terg0	4	>256	1	>2	>256	32	0.25	<0.25	0.5	<0.125	128	64
Site 2	452	BileV+	<0.125	0.25	<0.125	<0.125	0.5	0.5	<0.125	<0.125	0.25	<0.125	8	8
	457	BileV+	0.5	64	0.25	0.25	>128	16	<0.125	<0.0625	1	1	32	2
	460	BileV+	4	>64	<0.125	<0.125	>64	16	<0.125	<0.125	4	1	16	8
	467	BileV+	<0.125	<0.125	<0.125	<0.125	1	1	<0.125	<0.125	0.25	<0.125	8	32
	506	Terg0	>128	>128	1	1	>128	64	<0.125	0.25	1	<0.125	16	8
	511	Terg0	NA	>128	1	<0.03125	>128	NA	<0.125	<0.0625	1	1	NA	NA
	517	TergC+	16	32	128	16	0.125	1	0.03125	<0.25	2	<1	16	2
	671	Terg0	<0.125	<0.125	<0.125	<0.125	0.25	0.5	<0.125	<0.125	NA	NA	NA	NA
Site 3	462	Terg0	2	>128	0.5	NA	>128	64	0.25	NA	0.5	1	NA	>64
	591	Terg0	0.0625	<0.5	<0.5	0.015625	4	1	0.25	<0.25	0.5	<0.125	64	16
	593	Terg0	0.125	<0.5	<0.5	0.125	16	0.5	0.0625	<0.25	1	<0.125	128	16
	681	BileV+	<0.125	0.25	1	NA	2	2	NA	<0.125	0.25	<0.125	8	32
	683	BileV+	<0.125	<0.125	0.5	NA	0.5	1	NA	<0.125	<0.125	<0.125	4	16
	688	BileV+	4	>64	4	0.25	>64	1	1	<0.125	0.25	>0.125	4	16
	693	BileV+	1	32	1	0.25	>64	8	0.5	>0.125	0.5	>0.125	8	8
	695	BileV+	4	>64	0.25	0.25	>64	16	<0.125	<0.125	16	<0.125	16	16
	696	BileV+	>64	>64	0.5	0.25	>64	16	64	<0.125	0.5	<0.125	8	16
	697	BileV+	64	64	4	2	>64	64	0.25	<0.125	>64	>64	32	>64
	698	BileV+	>64	>64	0.5	0.5	>64	16	64	<0.125	1	<0.125	8	16
	728	BileV+	32	>64	8	<0.25	>64	32	0.25	<0.125	32	<0.125	16	32
	762	Terg0	2	32	2	0.25	>64	32	0.5	<0.125	0.25	<0.125	NA	>64
	772	Terg0	>4	>256	0.5	1	>256	NA	>4	>2	<0.5	<0.5	>256	NA
	778	Terg0	<0.25	<0.25	0.125	0.125	1	1	<0.125	0.125	0.5	0.5	16	2

	789	Terg0	4	128	<0.25	0.25	>128	32	2	<0.0625	0.5	<0.25	8	2
	790	Terg0	2	>64	0.5	1	>64	32	0.5	<0.125	64	<0.125	16	64
	791	Terg0	<0.125	0.25	<0.125	<0.125	0.25	0.5	<0.125	<0.125	1	<0.125	8	32
	793	Terg0	1	>64	0.25	0.5	>64	16	0.125	<0.125	4	<0.125	16	16
	871	TergC+	<0.5	<0.5	16	16	4	2	0.0625	<0.25?	0.5	<0.125	16	NA
	881	Terg0	<0.25	<0.25	<0.03125	0.25	1	2	<0.125	0.125	0.5	1	16	2
Site 4	**926**	BileV+	<0.25	0.25	<0.125	0.0625	<0.125	64	0.03125	<0.125	0.5	<0.125	8	>64
	935	BileV+	<0.125	0.25	<0.125	0.0625	0.5	1	0.125	<0.125	0.5	<0.125	16	16
	941	BileV+	<0.125	0.125	<0.125	<0.125	1	1	<0.125	<0.125	0.25	<0.125	16	16
	948	Terg0	<0.125	0.25	<0.125	<0.125	1	0.25	0.125	<0.125	0.25	<0.125	8	16

The minimum inhibitory concentrations were determined for E. coli isolates isolated from wastewater from four different sites at one hospital: Site 1: Radiology/Oncology; Site 2: Residential Site 3: Theatre/Emergency/Outpatient; and Site 4: Reference site. Isolates were recovered from various selective media: Tergitol-7 without additional antibiotics Terg0; Tergitol-7 with 1mg/L of ciprofloxacin TergC; Pseudomonas agar without antibiotics Ps0; bile aesculin with 4mg/L of vancomycin BileV+ and XLD with 1mg/L of ciprofloxacin XLD C+. Eight different antibiotics: cefepime (FEP), ceftazidime (CAZ), ciprofloxacin (CIP), levofloxacin (LEV), gentamicin (GEN), tobramycin (TOB), meropenem (MER), colistin (COL) and 4 other antimicrobials: chlorhexidine (CHL), triclosan (TRI), benzalkonium chloride (BNZ) and silver nitrate ($AgNO_3$) were used. Results were compared to the epidemiological cut-off values, displayed in the table as mg/L, from the European Committee on Antimicrobial Susceptibility Testing [118]. Resistance/sensitivity is indicated as the following colours: resistance - salmon; sensitive- green, grey, unable to be determined. Isolates displaying resistance against more than one family of antibiotics were deemed multidrug resistant and are indicated in dark red.

Appendix 5. Antimicrobial susceptibility profile of K. pneumoniae isolated from hospital wastewater

K. pneumoniae		Class	Cephalosporins		Fluoroquinolones		Aminoglycosides		Carbapenems		Polymyxin	Disinfectants			Heavy Metal
		Antibiotic	FEP	CAZ	CIP	LEV	GEN	TOB	MER	IMI	COL	CHL	TRI	BNZ	$AgNO_3$
		EUCAST breakpoint	0.008-0.125	0.032-0.5	0.008-0.125	0.032-0.25	0.125-4	0.25-2	0.008-0.5	0.064-1	0.125-2	64	NA	16	16
Site	Isolate	Plated from													
Site 1	314	TergC+	>128	>128	>32	8	32	>128	<0.25	NA	<0.25	8	>64	8	2
	326	TergC+	16	16	8	2	32	16	<0.25	NA	<0.25	1	<0.5	8	1
	345	TergC+	>64	>64	>64	32	>64	>64	<0.125	NA	0.5	4	<0.125	16	8
	348	TergC+	>64	>64	>64	32	>64	>64	0.5	NA	1	4	<0.125	16	8
	365	Terg0	64	16	2	4	128	4	<0.25	0.5	<0.25	16	2	NA	NA
	383	Terg0	32	>64	4	0.5	>64	64	<0.125	NA	<0.125	16	<0.125	8	8
Site 2	435	BileV+	0.25	0.5	<0.125	0.25	1	2	0.25	NA	1	16	>64	8	8
	475	Terg0	2	16	<0.125	0.25	<0.125	0.125	<0.125	NA	<0.125	0.25	<0.125	8	2
	477	Terg0	64	>128	8	2	>128	16	<0.25	0.25	<0.25	1	4	NA	NA
	482	Terg0	1	0.5	1	2	32	4	<0.25	NA	<0.25	8	<0.25	8	2
	483	Terg0	8	64	0.5	0.5	>64	4	0.25	NA	0.25	<0.125	<0.125	16	4
	484	Terg0	0.5	2	<0.125	0.25	>64	16	<0.125	NA	<0.125	32	<0.125	16	8
	485	Terg0	32	>64	0.25	<0.125	64	NA	<0.125	1	<0.125	NA	1	NA	8
	486	Terg0	<0.125	0.25	<0.125	<0.125	1	2	<0.125	NA	<0.125	8	16	16	8
	487	Terg0	16	64	2	2	>64	32	0.5	NA	0.25	4	2	32	64
	495	Terg0	2	>128	<0.25	<0.25	0.5	<0.25	<0.25	<0.25	<0.25	0.5	8	NA	NA
	499	Terg0	0.25	2	<0.125	<0.125	1	1	<0.125	NA	<0.125	2	0.25	8	16
	502	Terg0	16	>64	2	4	16	64	<0.125	NA	0.5	8	0.5	16	8
	504	Terg0	1	8	<0.125	<0.125	64	32	<0.125	<0.125	<0.25	1	<0.125	8	2
	505	Terg0	4	>64	0.5	0.5	8	4	<0.125	NA	<0.125	4	<0.125	8	8

	513	Terg0	0.25	4	<0.125	<0.125	64	8	<0.125	NA	<0.125	16	<0.125	16	16
	514	Terg0	2	>64	0.5	1	64	32	<0.125	NA	<0.25	32	64	8	8
	518	TergC+	32	>64	8	2	64	8	0.5	0.5	0.25	8	>1024	16	4
	534	Ps0	16	>64	1	2	64	4	<0.125	NA	<0.125	2	<0.125	16	8
Site 3	**694**	BileV+	0.5	>64	0.5	1	>64	16	0.5	NA	0.25	2	4	16	8
	755	BileV+	2	>128	0.5	0.5	>128	16	0.5	NA	<0.125	2	<0.125	8	8
	761	Terg0	4	>128	0.5	0.5	>128	32	0.5	1	0.25	<0.125	<0.25	8	4
	815	Terg0	>64	>64	0.25	1	16	64	0.5	NA	<0.125	1	<0.125	16	8
	816	Terg0	64	>64	<0.125	<0.125	8	64	<0.125	NA	<0.125	<0.125	<0.125	NA	8
	817	Terg0	<0.125	0.5	<0.125	<0.125	0.25	<0.125	<0.125	NA	<0.125	2	<0.125	8	4
	825	Terg0	<0.125	0.5	<0.125	<0.125	0.5	0.25	<0.125	NA	<0.125	8	<0.125	8	4
Site 4	**938**	BileV+	<0.125	<0.125	<0.125	<0.125	<0.125	0.25	<0.125	<0.125	<0.125	NA	<0.125	NA	8
	959	Terg0	<0.125	<0.125	<0.125	<0.125	0.5	0.5	<0.125	<0.125	<0.125	>64	<0.125	8	8
	1040	BileV+	<0.125	0.25	<0.125	<0.125	<0.125	<0.125	<0.125	<0.125	0.25	NA	<0.125	NA	4

The minimum inhibitory concentrations were determined for K. pneumoniae isolates isolated from wastewater from four different sites at one hospital: Site 1: Radiology/Oncology; Site 2: Residential Site 3: Theatre/Emergency/Outpatient; and Site 4: Reference site. Isolates were recovered from various selective media: Tergitol-7 without additional antibiotics Terg0; Tergitol-7 with 1mg/L of ciprofloxacin TergC; Pseudomonas agar without antibiotics Ps0; bile aesculin with 4mg/L of vancomycin BileV+ and XLD with 1mg/L of ciprofloxacin XLD C+. Eight different antibiotics: cefepime (FEP), ceftazidime (CAZ), ciprofloxacin (CIP), levofloxacin (LEV), gentamicin (GEN), tobramycin (TOB), meropenem (MER), colistin (COL) and 4 other antimicrobials: chlorhexidine (CHL), triclosan (TRI), benzalkonium chloride (BNZ) and silver nitrate ($AgNO_3$) were used. Results were compared to the epidemiological cut-off values, displayed in the table as mg/L, from the European Committee on Antimicrobial Susceptibility Testing [118]. Resistance/sensitivity is indicated as the following colours: resistance - salmon; sensitive- green, grey, unable to be determined. Isolates displaying resistance against more than one family of antibiotics were deemed multidrug resistant and are indicated in dark red.

Appendix 6. Antimicrobial susceptibility profile of K. oxytoca isolated from hospital wastewater

K. oxytoca		Class	Cephalosporins		Fluoroquinolones		Aminoglycosides		Carbapenems		Polymyxin	Disinfectants			Heavy Metal
		Antimicrobial	FEP	CAZ	CIP	LEV	GEN	TOB	MER	IMI	COL	CHL	TRI	BNZ	$AgNO_3$
		EUCAST breakpoint	0.008-0.125	0.032-0.5	0.008-0.125	0.032-0.25	0.25-2	0.25-2	0.064-1	0.064-1	0.125-2	64	0.25	32	32
Site	Isolate	Plated from													
Site 1	278	XLDC+	64	64	16	0.5	32	8	0.125	0.25	0.0625	0.25	0.25	16	4
	279	XLDC+	>64	64	4	1	>64	32	1	0.25	0.0625	2	0.25	16	2
	280	XLDC+	>64	>64	32	8	>64	32	2	0.25	0.25	8	4	16	8
	281	XLDC+	>8	8	4	1	>8	>8	2	0.25	0.125	4	0.5	8	4
	282	XLDC+	>64	>64	32	2	>64	16	0.125	<0.125	<0.125	0.25	<0.125	32	2
	283	XLDC+	>64	32	>64	1	>64	16	0.5	0.25	<0.125	4	<0.125	32	2
	285	XLDC+	64	64	4	1	64	16	<0.125	<0.125	<0.125	16	<0.125	16	4
	286	XLDC+	>64	>64	4	1	>64	16	0.5	0.25	<0.125	32	<0.125	32	4
	290	Terg0	32	>64	64	2	64	32	0.25	0.25	<0.125	16	<0.125	16	8
	298	Terg0	16	>64	0.5	0.25	>64	16	<0.125	<0.125	<0.125	4	<0.125	8	16
	299	Terg0	>64	64	2	2	64	32	2	0.5	<0.125	2	<0.125	8	4
	303	Terg0	>64	64	2	0.125	64	16	0.125	<0.125	<0.125	0.125	<0.125	16	8
	305	Terg0	256	128	8	32	256	16	0.5	0.5	0.0625	4	<0.125	8	>64
	325	TergC+	>64	>64	8	1	>64	32	<0.125	<0.125	<0.125	8	<0.125	16	16
	328	Terg0	512	256	2	1	256	64	<0.125	<0.125	0.25	2	0.25	8	8
	332	TergC+	>64	>64	32	0.5	>64	32	0.25	0.5	<0.125	16	<0.125	16	16
	333	TergC+	>64	>64	8	4	>64	>64	0.25	<0.125	0.25	16	0.25	32	32
	334	TergC+	16	1024	8	2	1024	32	<0.125	<0.125	0.25	8	0.5	16	16
	336	TergC+	>64	>64	4	2	>64	64	0.5	0.25	<0.125	8	0.5	16	8
	337	TergC+	>64	>64	8	1	>64	32	2	0.25	<0.125	32	0.25	16	16
	338	TergC+	8	>512	8	2	128	64	0.125	0.25	0.125	2	0.25	16	8
	341	TergC+	>64	>64	2	0.5	>64	32	0.25	0.25	0.125	8	<0.125	16	16
	356	BileV+	>1024	>1024	64	16	512	64	0.5	0.5	0.0313	>64	0.5	256	>64
	363	Terg0	>64	>64	4	1	>64	32	<0.125	0.5	<0.125	0.25	2	16	1
	366	Terg0	>64	>64	4	2	>64	64	1	0.5	<0.125	32	8	32	4
	370	Terg0	16	>64	4	1	>64	8	0.5	<0.125	<0.125	32	<0.125	32	4
	372	Terg0	>64	512	64	8	32	16	1	0.5	0.125	32	0.5	8	4
	373	Terg0	>64	>64	8	1	>64	32	2	0.25	<0.125	>64	4	32	4

	375	Terg0	>64	64	64	32	2	32	1	<0.125	<0.125	16	<0.125	32	4
	386	BileV+	>64	>64	4	0.5	>64	64	<0.125	0.125	<0.125	0.5	<0.125	16	16
	393	BileV+	8	>64	2	1	64	>64	<0.125	0.125	<0.125	0.5	<0.125	16	8
	405	Ps0	8	>64	16	2	>64	16	<0.125	<0.065	<0.125	4	<0.125	16	8
	406	Ps0	8	>64	4	1	32	4	<0.125	0.25	<0.125	4	<0.125	32	4
	410	Ps0	8	>64	4	2	32	16	<0.125	<0.125	<0.125	2	<0.125	16	8
	426	BileV+	>64	>64	2	2	>64	32	0.125	0.25	0.25	4	<0.1255	16	32
	427	BileV+	64	>64	0.5	1	>64	64	<0.125	0.5	<0.125	16	16	16	16
Site 2	**429**	BileV+	<0.125	<0.125	<0.125	<0.125	2	0.5	<0.125	0.5	<0.125	4	0.125	16	8
	431	BileV+	<0.125	<0.125	<0.125	<0.125	0.25	1	<0.125	0.25	<0.125	16	<0.125	8	16
	447	BileV+	<0.125	<0.125	<0.125	<0.125	1	1	<0.125	0.25	<0.125	4	<0.125	8	16
	459	BileV+	0.015625	0.25	0.015625	<0.125	0.5	0.5	0.015625	0.5	0.125	2	512	16	16
	462	BileV+	<0.125	<0.125	<0.125	0.03125	<0.125	0.25	<0.125	<0.125	<0.125	4	<0.125	8	16
	465	BileV+	0.125	0.25	<0.125	0.25	0.5	<0.125	0.25	0.25	<0.125	4	0.25	16	8
	466	BileV+	<0.125	0.25	0.0625	<0.125	0.25	<0.125	<0.125	0.5	0.25	<0.25	0.125	8	8
	480	Terg0	1	1024	0.5	0.5	1024	8	0.125	1	0.25	8	0.25	16	16
	488	Terg0	0.5	128	0.5	2	128	8	0.125	0.5	0.125	4	0.125	8	>64
	669	Terg0	<0.125	<0.125	<0.125	<0.125	0.25	1	<0.125	<0.125	<0.125	1	<0.125	8	16
	672	Terg0	<0.125	<0.125	<0.125	<0.125	0.5	0.5	<0.125	0.25	<0.125	2	<0.125	8	2
Site 3	**599**	XLD C+	0.25	<0.125	0.25	1	0.25	2	<0.125	0.25	<0.125	1	2	2	0.25
	600	XLD C+	NA	NA	NA	NA	NA	NA	NA	NA	NA	2	8	2	4
	601	XLD C+	>64	>64	>64	16	>64	32	0.5	1	<0.25	16	4	16	8
	602	XLD C+	NA	NA	NA	NA	NA	NA	NA	NA	NA	2	16	4	>64
	605	XLD C+	32	512	32	16	256	8	1	0.25	0.125	4	0.25	8	1
	606	TergC+	2	>64	4	2	32	16	0.25	0.5	<0.125	2	0.5	8	8
	610	TergC+	2	>64	2	1	16	4	<0.125	0.5	0.125	4	0.25	8	8
	611	TergC+	1024	1024	16	4	256	64	4	0.5	0.125	32	1	16	4
	618	TergC+	>64	>64	32	8	>64	32	2	0.5	0.125	16	4	16	4
	619	TergC+	512	32	32	8	>64	4	1	0.5	0.125	4	0.5	8	0.03125
	621	TergC+	32	512	16	8	128	32	<0.125	0.5	0.125	4	0.5	8	8
	622	TergC+	16	>64	8	2	>64	32	0.25	0.5	<0.125	1	0.5	16	16
	623	TergC+	32	512	16	8	64	16	0.125	0.5	0.0625	32	0.5	8	4
	624	TergC+	>64	>64	32	4	>64	32	<0.125	0.5	<0.125	16	1	16	>64
	625	TergC+	0.5	16	8	<0.125	0.25	32	<0.125	0.5	<0.125	0.25	0.5	8	4
	630	TergC+	>64	>64	16	8	>64	64	<0.125	0.5	0.25	32	0.5	16	4
	727	BileV+	0.5	8	<0.125	0.015625	128	16	>0.125	0.25	<0.125	2	0.5	16	16

	751	Terg0	16	>64	16	2	>64	8	0.25	0.5	<0.125	2	0.5	16	32
	777	Terg0	64	>64	64	16	32	32	1	0.5	<0.125	2	1	16	16
	805	Terg0	4	>64	4	1	16	4	<0.125	0.5	<0.125	2	0.5	8	16
	806	Terg0	16	>64	8	2	>64	32	0.5	0.5	<0.125	0.5	0.5	16	16
	807	Terg0	8	32	0.5	<0.125	>64	16	0.125	2	<0.125	8	0.25	8	8
	809	Terg0	0.5	0.5	2	1	1	0.5	0.5	0.5	<0.125	16	>1024	16	16
	818	Terg0	4	8	0.5	1	0.125	0.25	0.125	1	<0.125	2	<0.125	16	8
	819	Terg0	2	4	0.25	0.25	64	0.25	0.25	0.25	0.125	0.25	0.5	8	8
	821	Terg0	<0.125	0.25	0.03125	<0.125	1	0.5	0.5	0.5	0.125	8	0.25	16	8
	822	Terg0	8	>64	16	4	>64	>64	0.5	0.5	<0.125	4	1	16	8
Site 4	**831**	Terg0	0.125	0.125	0.0625	<0.125	<0.125	<0.125	<0.125	0.25	0.25	<0.25	0.25	8	4
	832	Terg0	<0.125	0.125	<0.125	<0.125	0.25	0.5	<0.125	0.5	<0.125	2	<0.125	16	32
	839	TergC+	0.125	0.25	0.0625	<0.125	1	0.5	<0.125	0.5	0.25	<0.125	0.125	16	4
	848	Ps0	<0.125	16	1	0.5	0.5	0.125	<0.125	0.25	0.25	4	0.25	32	4
	849	Ps0	<0.125	0.25	0.5	0.5	1	0.125	<0.125	0.25	0.125	4	<0.125	16	8
	880	Terg0	0.5	0.25	0.015625	0.015625	1	0.125	0.0625	0.25	0.125	<0.25	0.25	8	2
	885	Terg0	<0.125	<0.125	0.25	<0.125	0.5	0.5	0.03125	0.25	0.5	4	1	8	16
	887	Terg0	<0.125	<0.125	<0.125	<0.125	1	0.5	0.125	0.5	<0.125	2	<0.125	16	8
	921	Terg0	0.5	16	<0.125	<0.125	1	1	<0.125	0.5	<0.125	2	<0.125	16	16
	933	BileV+	<0.125	0.125	0.03125	<0.125	0.25	0.125	<0.125	0.25	0.25	8	<0.125	16	8
	936	BileV+	<0.125	<0.125	0.5	2	0.5	0.5	<0.125	0.25	<0.125	0.5	<0.125	8	8
	937	BileV+	0.125	0.25	<0.125	0.03125	1	0.25	<0.125	0.5	0.125	8	0.25	8	16
	944	Terg0	0.125	0.25	0.0625	<0.125	0.5	1	<0.125	0.5	0.25	4	0.25	16	8
	947	Terg0	2	<0.125	<0.125	<0.125	<0.125	<0.125	<0.125	0.5	<0.125	2	<0.125	16	32
	960	Terg0	<0.125	<0.125	<0.125	<0.125	1	<0.125	<0.125	0.25	<0.125	2	<0.125	16	16
	961	Terg0	<0.125	0.5	<0.125	<0.125	1	0.5	<0.125	0.5	<0.125	2	<0.125	16	16

The minimum inhibitory concentrations were determined for K. oxytoca isolates isolated from wastewater from four different sites at one hospital: Site 1: Radiology/Oncology; Site 2: Residential Site 3: Theatre/Emergency/Outpatient; and Site 4: Reference site. Isolates were recovered from various selective media: Tergitol-7 without additional antibiotics Terg0; Tergitol-7 with 1mg/L of ciprofloxacin TergC; Pseudomonas agar without antibiotics Ps0; bile aesculin with 4mg/L of vancomycin BileV+ and XLD with 1mg/L of ciprofloxacin XLD C+. Eight different antibiotics: cefepime (FEP), ceftazidime (CAZ), ciprofloxacin (CIP), levofloxacin (LEV), gentamicin (GEN), tobramycin (TOB), meropenem (MER), colistin (COL) and 4 other

antimicrobials: chlorhexidine (CHL), triclosan (TRI), benzalkonium chloride (BNZ) and silver nitrate ($AgNO_3$) were used. Results were compared to the epidemiological cut-off values, displayed in the table as mg/L, from the European Committee on Antimicrobial Susceptibility Testing [118]. Resistance/sensitivity is indicated as the following colours: resistance - salmon; sensitive- green, grey, unable to be determined. Isolates displaying resistance against more than one family of antibiotics were deemed multidrug resistant and are indicated in dark red.

Appendix 7. Virulence genes found in K. oxytoca isolated from hospital wastewater

Gene	Accession Number	Isolate							
		356	459	480	488	611	623	809	880
aslA	AAG10151	0	0	81.36	81.36	0	0	0	81.36
astA	BAA94855	88.7	0	0	0	0	0	0	0
clfA	NP_645581	0	0	84.61	81.49	79.54	81.15	80.54	82.99
clfB	NP_647368	0	77.21	76.47	76.47	0	0	0	0
entA	NP_752614	80.58	80.08	80.08	80.08	80.36	80.36	80.58	81.18
entB	NP_752613	81.61	80.56	80.67	80.67	80.91	80.91	81.14	80.67
entE	NP_752612	76.72	76.91	77.04	77.04	77.65	77.65	76.94	77.04
entS	NP_752609	75.76	77.38	77.34	77.34	76.74	76.74	76.09	77.34
fepA	NP_752600	78.95	79.36	79.36	79.36	79.22	79.22	79.11	79.32
fepB	NP_752610	78.93	78.34	78.55	78.55	78.21	78.21	78.81	78.34
fepC	NP_752606	79.53	79.28	79.28	79.28	79.92	79.92	79.66	79.28
fepD	NP_752608	77.71	76.79	76.99	76.99	77.14	77.14	77.19	76.99
fepG	NP_752607	78.64	78.71	78.82	78.82	78	78	78.48	78.94
fimB	NP_757239	75.23	0	0	0	0	0	0	0
fimC	NP_757243	76.91	75.84	76.01	76.01	75.69	75.69	0	76.01
fimD		0	75.13	0	0	0	78.44	0	0
fimG	NP_757247	75.72	75.55	75.55	75.55	0	0	75.95	75.55
fyuA	NP_405467	90.45	90.16	0	0	89.47	89.47	90.45	76.56
gmhA/lpcA	NP_439337	77.23	76.34	76.34	76.34	75.67	75.67	76.79	79.23
gspG	YP_404603	79.17	79.23	79.23	79.23	0	0	79.71	0
irp1	NP_405471	87.04	86.96	0	0	85.96	85.96	87.21	0
irp2	NP_405472	87.09	87.26	0	0	85.47	85.47	87.19	0
katB	YP_096397	0	81.03	81.03	81.03	0	0	0	81.03

mgtB	NP_462662	80.69	79.97	79.97	79.97	79.74	79.74	80.57	80.04
mgtC	NP_462663	75.40	0	0	0	0	0	75.40	0
ompA	AAF37887	83.01	83.38	83.38	83.38	82.63	82.63	83.29	83.29
pilW	YP_001007658	0	75.14	75.14	75.14	0	0	0	75.69
pla	NP_395233	0	0	75.13	75.13	78.71	78.71	0	75.13
sdrC	NP_645333	0	0	84.44	79.44	0	80.34	85.51	84.83
sdrD	NP_645334	0	86.14	0	83.59	0	0	80.88	0
tcpI		0	0	0	0	75.34	75.34	0	0
waaC	NP_253698	78.65	78.65	80.21	80.21	77.08	77.08	79.17	78.65
xcpR	NP_251793	0	75.76	75.73	75.73	0	0	0	75.73
yagW/ecpD	NP_286007	79.13	79.37	79.37	79.37	79.35	79.35	79.04	79.37
yagX/ecpC	NP_286008	78.63	78.08	78	78	78.56	78.56	78.67	78.08
yagY/ecpB	NP_286009	77.58	77.58	77.58	77.58	78.47	78.47	77.28	77.58
yagZ/ecpA	NP_286010	82.88	82.96	82.96	82.96	82.88	82.88	83.73	82.96
ybtA	NP_405473	89.79	89.69	0	0	89.17	89.17	90	0
ybtE	NP_405468	85.61	85.37	0	0	84.26	84.26	85.68	0
ybtP	NP_405474	88.02	87.85	0	0	85.75	85.75	88.19	0
ybtQ	NP_405475	87.47	87.74	0	0	87.41	87.41	87.58	0
ybtS	NP_405477	87.79	87.79	0	0	85.82	85.82	87.71	0
ybtT	NP_405469	84.95	84.45	0	0	84.77	84.77	84.83	0
ybtU	NP_405470	85.71	86.47	0	0	85.35	85.35	86.45	0
ybtX	NP_405476	87.67	87.20	0	0	83.47	83.47	87.82	0

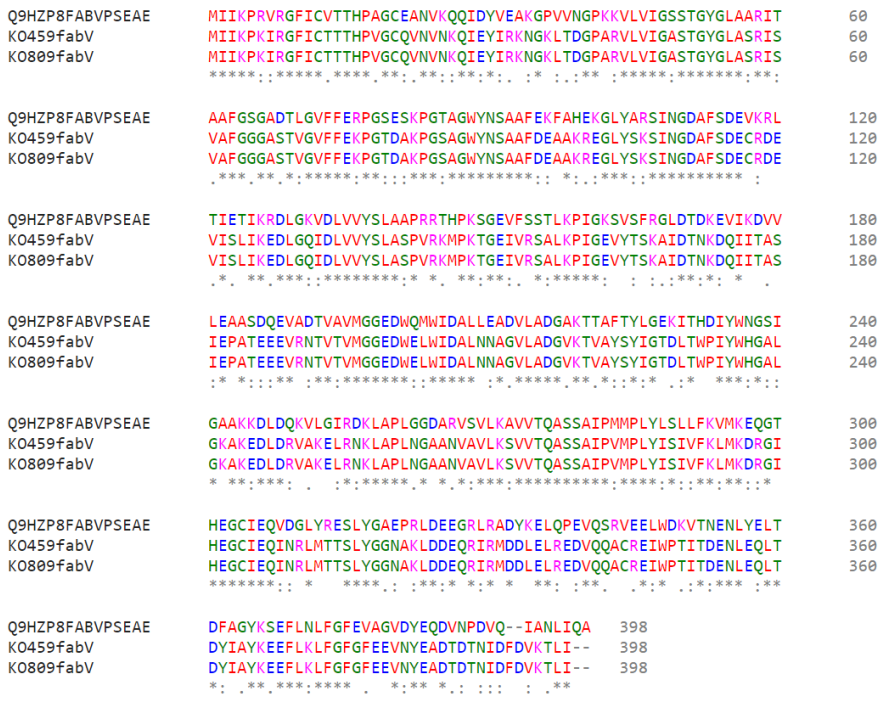
Table indicates the % matched with Virulence genes according to Prokka database.

Appendix 8. Triclosan resistance determinants found in K. oxytoca isolated from hospital wastewater

Isolate	356	459	480	488	611	623	809	880
Triclosan MIC (mg/L)	0.5	512	0.25	0.125	1	0.5	>1024	0.25
abeM	*abeM*	.	.	.	.	.	.	.
acrA*	*acrA*	*acrA*	*acrA*	*acrA*	*acrA*	*acrA*	*acrA*	*acrA*
acrB*	*acrB_1*	*acrB_1*	*acrB_1*	*acrB_1*	*acrB_1*	*acrB_1*	*acrB_1*	*acrB_1*
	acrB_2	*acrB_2*	*acrB_2*	*acrB_2*	*acrB_2*	*acrB_2*	*acrB_2*	*acrB_2*
	.	*acrB_3*	.	.	.	.	*acrB_3*	.
fabA	*fabA*	*fabA*	*fabA*	*fabA*	*fabA*	*fabA*	*fabA*	*fabA*
fabB	*fabB*	*fabB*	*fabB*	*fabB*	*fabB*	*fabB*	*fabB*	*fabB*
fabD	*fabD_1*	*fabD_1*	*fabD_1*	*fabD_1*	*fabD_1*	*fabD_1*	*fabD_1*	*fabD_1*
	fabD_2	*fabD_2*	*fabD_2*	*fabD_2*	*fabD_2*	*fabD_2*	*fabD_2*	*fabD_2*
fabF	*fabF_1*	*fabF_1*	*fabF_1*	*fabF_1*	*fabF_1*	*fabF_1*	*fabF_1*	*fabF_1*
	fabF_2	*fabF_2*	*fabF_2*	*fabF_2*	*fabF_2*	*fabF_2*	*fabF_2*	*fabF_2*
	.	*fabF_3*	*fabF_3*	*fabF_3*	*fabF_3*	*fabF_3*	.	*fabF_3*
	.	*fabF_4*	*fabF_4*	*fabF_4*	.	.	.	*fabF_4*
fabG	*fabG_1*	*fabG_1*	*fabG_1*	*fabG_1*	*fabG_1*	*fabG_1*	*fabG_1*	*fabG_1*
	fabG_2	*fabG_2*	*fabG_2*	*fabG_2*	*fabG_2*	*fabG_2*	*fabG_2*	*fabG_2*
	fabG_3	*fabG_3*	*fabG_3*	*fabG_3*	*fabG_3*	*fabG_3*	*fabG_3*	*fabG_3*
	fabG_4	*fabG_4*	*fabG_4*	*fabG_4*	*fabG_4*	*fabG_4*	*fabG_4*	*fabG_4*
	fabG_5	*fabG_5*	*fabG_5*	*fabG_5*	*fabG_5*	*fabG_5*	*fabG_5*	*fabG_5*
	fabG_6	*fabG_6*	*fabG_6*	*fabG_6*	*fabG_6*	*fabG_6*	*fabG_6*	*fabG_6*
	fabG_7	*fabG_7*	*fabG_7*	*fabG_7*	*fabG_7*	*fabG_7*	*fabG_7*	*fabG_7*
	fabG_8	*fabG_8*	*fabG_8*	*fabG_8*	*fabG_8*	*fabG_8*	*fabG_8*	*fabG_8*
	fabG_9	*fabG_9*	*fabG_9*	*fabG_9*	*fabG_9*	*fabG_9*	*fabG_9*	*fabG_9*
	fabG_10	*fabG_10*	*fabG_10*	*fabG_10*	*fabG_10*	*fabG_10*	*fabG_10*	*fabG_10*
	fabG_11	*fabG_11*	*fabG_11*	*fabG_11*	*fabG_11*	*fabG_11*	*fabG_11*	*fabG_11*
	.	.	.	.	*fabG_12*	*fabG_12*	.	.
	.	.	.	.	*fabG_13*	*fabG_13*	.	.
	.	.	.	.	*fabG_14*	*fabG_14*	.	.
fabH	*fabH*	*fabH*	*fabH*	*fabH*	*fabH*	*fabH*	*fabH*	*fabH*
fabI	*fabI*	*fabI*	*fabI*	*fabI*	*fabI*	*fabI*	*fabI*	*fabI*
fabL/yga	.	.	.	.	.	.	*fabL*	.
fabR	*fabR*	*fabR*	*fabR*	*fabR*	*fabR*	*fabR*	*fabR*	*fabR*
fabV	.	***fabV***	.	.	.	.	***fabV***	.
fabZ	*FabZ*	*fabZ*	*fabZ*	*fabZ*	*fabZ_1*	*fabZ_1*	*fabZ*	*fabZ*
	.	.		.	*fabZ_2*	*fabZ_2*	.	.
kpnF	.	.	.	.	.	.	.	.
toIC	.	.	.	.	.	.	.	.
triA	.	.	.	.	.	.	.	.
triB	.	.	.	.	.	.	.	.
triC	.	.	.	.	.	.	.	.

Table showing the absence of presence of resistance determinants against triclosan. Grey boxes indicate the absence of a gene. The triclosan resistant gene fabV is indicated in red writing. Triclosan resistance genes according to the Bacmet database (http://bacmet.biomedicine.gu.se/).For phenotypic comparison, the minimum inhibitory concentration (MIC)(mg/L) has been included. Triclosan sensitivity (green) and triclosan resistance (red) was based on the ECOFF of 0.25mg/L as determined by the MIC of 88 isolates and using the ECOFFFinderXL2010.xlsm tool (https://clsi.org/meetings/microbiology/ecoffinder/).**AcrA and AcrB are genomically encoded, upregulation or mutation is associated with triclosan resistance.*

Appendix 9. The multiple sequence alignment of fabV from P. aeruginosa and fabV isolated from hospital wastewater K. oxytoca



Strictly conserved residues are makred with an asteriks (*). Conserved and semiconserved substitutions are represented by (:) and (.), dashes represent gaps. The Vibrio sp., fabV sequence was obtained from the National Center for Biotechnology Information Protein (NCBI) databse by the use of the following accesion number Q9HZP8. Ailgnment was performed using ClsutalW at http://custalw.genome.jp/

Appendix 10. The multiple sequence alignment of fabV from V. cholerae and fabV isolated from wastewater K. oxytoca

```
Q9KRA3FABV1VIBCH      MIIKPKIRGFICTTTHPVGCEANVKEQIAYTKAQGPIKNAPKRVLVVGSSSGYGLSSRIA      60
KO459fabV             MIIKPKIRGFICTTTHPVGCQVNVNKQIEYIRKNGKLTDGPARVLVIGASTGYGLASRIS      60
KO809fabV             MIIKPKIRGFICTTTHPVGCQVNVNKQIEYIRKNGKLTDGPARVLVIGASTGYGLASRIS      60
                      ********************:.**:.** * : :* :.:.* ****:*:*:****:***:

Q9KRA3FABV1VIBCH      AAFGGGAATIGVFFEKPGTDKKPGTAGFYNAAAFDKLAHEAGLYAKSLNGDAFSNEAKQK      120
KO459fabV             VAFGGGASTVGVFFEKPGTDAKPGSAGWYNSAAFDEAAKREGLYSKSINGDAFSDECRDE      120
KO809fabV             VAFGGGASTVGVFFEKPGTDAKPGSAGWYNSAAFDEAAKREGLYSKSINGDAFSDECRDE      120
                      .******:*:********** ***:**:**:****: *:. ***:**:******:*.:::

Q9KRA3FABV1VIBCH      AIELIKQDLGQIDLVVYSLASPVRKMPDTGELVRSALKPIGETYTSTAVDTNKDVIIEAS      180
KO459fabV             VISLIKEDLGQIDLVVYSLASPVRKMPKTGEIVRSALKPIGEVYTSKAIDTNKDQIITAS      180
KO809fabV             VISLIKEDLGQIDLVVYSLASPVRKMPKTGEIVRSALKPIGEVYTSKAIDTNKDQIITAS      180
                      .*.***:********************.***:**********.***.*:***** ** **

Q9KRA3FABV1VIBCH      VEPATEQEIADTVTVMGGQDWELWIQALEEAGVLAEGCKTVAYSYIGTELTWPIYWDGAL      240
KO459fabV             IEPATEEEVRNTVTVMGGEDWELWIDALNNAGVLADGVKTVAYSYIGTDLTWPIYWHGAL      240
KO809fabV             IEPATEEEVRNTVTVMGGEDWELWIDALNNAGVLADGVKTVAYSYIGTDLTWPIYWHGAL      240
                      :*****:*: :*******:******:**::*****:* **********:*******.***

Q9KRA3FABV1VIBCH      GRAKMDLDRAATALNEKLAAKGGTANVAVLKSVVTQASSAIPVMPLYIAMVFKKMREQGV      300
KO459fabV             GKAKEDLDRVAKELRNKLAPLNGAANVAVLKSVVTQASSAIPVMPLYISIVFKLMKDRGI      300
KO809fabV             GKAKEDLDRVAKELRNKLAPLNGAANVAVLKSVVTQASSAIPVMPLYISIVFKLMKDRGI      300
                      *:** ****.*. *.:***  .*:************************::*** *:::*:

Q9KRA3FABV1VIBCH      HEGCMEQIYRMFSQRLYKEDGSAPEVDDHNRLRLDDWELRDDIQQHCRDLWPQITTENLR      360
KO459fabV             HEGCIEQINRLMTTSLYGG---NAKLDDEQRIRMDDLELREDVQQACREIWPTITDENLE      357
KO809fabV             HEGCIEQINRLMTTSLYGG---NAKLDDEQRIRMDDLELREDVQQACREIWPTITDENLE      357
                      ****:*** *:::  **       ::**.:*:*:** ***:*:** **::** ** ***.

Q9KRA3FABV1VIBCH      ELTDYDMYKEEFIKLFGFGIEGIDYDADVNPEVEFDVIDIE 401
KO459fabV             QLTDYIAYKEEFLKLFGFGFEEVNYEADTDTNIDFDVKTLI 398
KO809fabV             QLTDYIAYKEEFLKLFGFGFEEVNYEADTDTNIDFDVKTLI 398
                      :****  *****:******:* ::*:**.: :::***  :
```

Strictly conserved residues are makred with an asteriks (*). Conserved and semiconserved substitutions are represented by (:) and (.), dashes represent gaps. The Vibrio sp., fabV sequence was obtained from the National Center for Biotechnology Information Protein (NCBI) databse by the use of the following accesion number WP_000584512.1. Ailgnment was performed using ClsutalW at http://custalw.genome.jp/

Appendix 11. Heavy metal resistance determinants identified from K. oxytoca isolated from hospital wastewater

Gene	Isolate							
	356	**459**	**480**	**488**	**611**	**623**	**809**	**880**
copA	*copA_1*	*copA_1*	*copA_1*	*copA_1*	*copA_1*	*copA_1*	*copA_1*	*copA_1*
	copA_2	*copA_2*	*copA_2*	*copA_2*	*copA_2*	*copA_2*	*copA_2*	*copA_2*
copB	*copB*	*copB*	*copB*	*copB*	*copB*	*copB*	*copB*	*copB*
cueA	.	.	.	.	.	.	.	.
cusA	*cusA_1*	*cusA_1*	*cusA_1*	*cusA_1*	*cusA_1*	*cusA_1*	*cusA_1*	*cusA_1*
	cusA_2	*cusA_2*	*cusA_2*	*cusA_2*	*cusA_2*	*cusA_2*	*cusA_2*	*cusA_2*
cusB	*cusB_1*	*cusB_1*	*cusB_1*	*cusB_1*	*cusB_1*	*cusB_1*	*cusB_1*	*cusB_1*
	cusB_2	*cusB_2*	*cusB_2*	*cusB_2*	*cusB_2*	*cusB_2*	*cusB_2*	*cusB_2*
cusC	*cusC_1*	*cusC_1*	*cusC_1*	*cusC_1*	*cusC_1*	*cusC_1*	*cusC_1*	*cusC_1*
	cusC_2	*cusC_2*	*cusC_2*	*cusC_2*	*cusC_2*	*cusC_2*	*cusC_2*	*cusC_2*
	cusC_3	*cusC_3*	*cusC_3*	*cusC_3*	.	.	.	*cusC_3*
cusF/cusX	*cusF_1*	*cusF_1*	*cusF*	*cusF*	*cusF*	*cusF*	*cusF_1*	*cusF_1*
	cusF_2	*cusF_2*	.	.	.	.	*cusF_2*	*cusF_2*
cusR/ylcA	*cusR*	*cusR*	*cusR*	*cusR*	*cusR*	*cusR*	*cusR*	*cusR*
cusS	*cusS_1*	*cusS_1*	*cusS_1*	*cusS_1*	*cusS_1*	*cusS_1*	*cusS_1*	*cusS_1*
	cusS_2	*cusS_2*	*cusS_2*	*cusS_2*	*cusS_2*	*cusS_2*	*cusS_2*	*cusS_2*
	.	.	*cusS_3*	*cusS_3*	*cusS_3*	*cusS_3*	*cusS_3*	*cusS_3*
pcoE	*pcoE*	*pcoE*	*pcoE_1*	*pcoE_1*	*pcoE_1*	*pcoE_1*	*pcoE*	*pcoE*
			pcoeE_2	*pcoeE_2*	*pcoeE_2*	*pcoeE_2*	.	
silA	.	.	.	.	.	.	.	.
silB	.	.	.	.	.	.	.	.
silC	.	.	.	.	.	.	.	.
silE	*silE_1*	*silE_1*	*silE_1*	*silE_1*	*silE_1*	*silE_1*	*silE_1*	*silE_1*
	silE_2	*silE_2*	*silE_2*	*silE_2*	*silE_2*	*silE_2*	*sileE_2*	*silE_2*
silF	.	.	.	.	.	.	.	.
silP	*silP*	*silP*	*silP*	*silP*	*silP*	*silP*	*silP*	*silP*
silR	.	.	.	.	.	.	.	.
silS	.	.	.	.	.	.	.	.

Table indicates the presence of common silver nitrate resistance genes according to the Bacmet database (http://bacmet.biomedicine.gu.se/).

Appendix 12. Multiple sequence alignment of silE and silP for Klebsiella oxytoca isolated from wastewater

```
KO809silE1      MKNIVLASVLGLSLISTAWATETVNIHERVNNAQAPAHQMQSSSTPAAIQGAAPRMAGMD   60
KO459silE1      MKNIVLASLLGFGLISSAWATETVNIHDRVNNAQAPAHQMQSAAAPAAIQGAAPRMAGMD   60
KO480silE1      -KNIVLASLLGFGLISSAWATETVNIHDRVNNAQAPAHQMQSAAAPAAIQGAAPRMAGMD   59
KO488silE1      MKNIVLASLLGFGLISSAWATETVNIHDRVNNAQAPAHQMQSAAAPAAIQGAAPRMAGMD   60
KO356silE1      MKNIVLASLLGFSLISSAWATETVNIHERVNNAQAPAHQMQSAAAPAVIQGTAPRMTGMD   60
KO611silE1      MKNIVLASLLGFGLISSAWATETVNIHDRVNNAQAPAHQMQSAAAPVGIQGTAPRMTGMD   60
KO623silE1      MKNIVLASLLGFGLISSAWATETVNIHDRVNNAQAPAHQMQSAAAPVGIQGTAPRMTGMD   60
KO880silE1      MKNIVLASLLGFGLISSAWATETVNIHDRVNNAQAPAHQMQSAAAPVGIQGTAPRMTGMD   60
                 *******:**:.***:**********:**************:::*. ***:****:***

KO809silE1      QHEQAIIVHETMNNGSADAHKKMAESHQKMMGTGTVNASRPATSFAAMNEHERAAVAHEF   120
KO459silE1      QHEQAIIVHETMNNGSADAHKKMAESHQKMMGTGTVNASRPATSFAAMNEHERAAVAHEF   120
KO480silE1      QHEQAIIVHETMNNGSADAHKKMAESHQKMMGTGTVNASRPATSFAAMNEHERAAVAHEF   119
KO488silE1      QHEQAIIVHETMNNGSADAHKKMAESHQKMMGTGTVNASRPATSFAAMNEHERAAVAHEF   120
KO356silE1      QHEQAIIAHETMTNESADAHQKMVESHQRMMGSQTVSPTGPSKSFAAMNEHERAAVAHEF   120
KO611silE1      QHEQAIIAHETMTNGSADAHQKMVESHQKMMGNNTVSTTVPSTSYAAMNEHERAAVAHEF   120
KO623silE1      QHEQAIIAHETMTNGSADAHQKMVESHQKMMGNNTVSTTVPSTSYAAMNEHERAAVAHEF   120
KO880silE1      QHEQAIIAHETMTNGSADAHQKMVESHQKMMGNNTVSTTVPSTSYAAMNEHERAAVAHEF   120
                 *******.****.* *****:**.****:***. **. : *:.*:***************

KO809silE1      TNNGQSGPHQAMADAHRRMINAG 143
KO459silE1      TNNGQSGPHQAMADAHRRMINAG 143
KO480silE1      TNNGQSGPHQAMADAHRRMINAG 142
KO488silE1      TNNGQSGPHQAMADAHRRMINAG 143
KO356silE1      MNNGQSGPHQAMAEAHRRMLNAG 143
KO611silE1      MNNGQSGPHQAMAEAHRRMINAG 143
KO623silE1      MNNGQSGPHQAMAEAHRRMINAG 143
KO880silE1      MNNGQSGPHQAMAEAHRRMINAG 143
                 ************:*****:***
```

a.

```
KO356silP       MKNDNAVEHNNQTASEQTSSPDESHALHKVRDPVCGMVILPDKAHSSIRYQDHQLYFCSA   60
KO809silP       MKNDNAVEHNNQTASEQTSSPDESHALHKVRDPVCGMVILPDKAHSSIRYQDHQLYFCSA   60
KO880silP       MKNDNAVQHNNQTASEQTLSPDEGHVLHKVRDPVCGMAILPDRAHSSIRYQDHQLYFCSA   60
KO459silP       MKNDNAVQHNNQTASEQTLSPDESHVLHKVRDPVCGMAILPDRAHSSIRYQDHQLYFCSA   60
KO480silP       MKNDNAVQHNNQTASEQTLSPDESHVLHKVRDPVCGMAILPDRAHSSIRYQDHQLYFCSA   60
KO488silP       MKNDNAVQHNNQTASEQTLSPDESHVLHKVRDPVCGMAILPDRAHSSIRYQDHQLYFCSA   60
KO611silP       MKNDNAVQHNNQTASEQTLSPDEGHVLHKVRDPVRGMAILPDRAHSSIRYQDHQLYFCSA   60
KO623silP       MKNDNAVQHNNQTASEQTLSPDEGHVLHKVRDPVRGMAILPDRAHSSIRYQDHQLYFCSA   60
                 *******:********** ****.*.******** **.****:*****************

KO356silP       SCESKFKAHPDHYFTEDASEH----HHHHDHHEVSPDKIKQSHRQAEKEISEGVWTCPMH   116
KO809silP       SCESKFKAHPDHYFTEDASEH----HHHHDHHEVSPDKIKQSHRQAEKEISEGVWTCPMH   116
KO880silP       SCESKFKAHPDRYLTEDASEHSH--HHHHDHHEVSPDQIKQPHNQAEKENSEGVWTCPMH   118
KO459silP       SCESKFKAHPDRYLTEDASEHSHHHHHHHHDHHEVSPDQIKQPHHQAEKENSEGVWTCPMH   120
KO480silP       SCESKFKAHPDRYLTEDASEHSHHHHHHHDHHEVSPDQIKQPHHQAEKENSEGVWTCPMH   120
KO488silP       SCESKFKAHPDRYLTEDASEHSHHHHHHHDHHEVSPDQIKQPHHQAEKENSEGVWTCPMH   120
KO611silP       SCESKFKAHPDRYLTEDASEHSH--HHHHDHHEVSPDQIKQPHNQAEKENSEGVWTCPMH   118
KO623silP       SCESKFKAHPDRYLTEDASEHSH--HHHHDHHEVSPDQIKQPHNQAEKENSEGVWTCPMH   118
                 **********:*:*******    ************:*** *.***** **********

KO356silP       PEIRRSGPGSCPVCGMALEPLVATASTGTSDELRDMTRRFWLGLLLAFPVLILEMGSHLF   176
KO809silP       PEIRRSGPGSCPVCGMALEPLVATASTGTSDELRDMTRRFWLGLLLAFPVLILEMGSHLF   176
KO880silP       PEIRRSGPGSCPVCGMALEPLVATASTGPSDELHDMTRRFWLGLLLAFPVLVLEMGSHLF   178
KO459silP       PEIRRSGPGSCPVCGMALEPLVATASTGPSDELRDMTRRFWLGLLLAFPVLVLEMGSHLF   180
KO480silP       PEIRRSGPGSCPVCGMALEPLVATASTGPSDELRDMTRRFWLGLLLAFPVLVLEMGSHLF   180
KO488silP       PEIRRSGPGSCPVCGMALEPLVATASTGPSDELRDMTRRFWLGLLLAFPVLVLEMGSHLF   180
KO611silP       PEIRRSGPGSCPVCGMALEPLVATASTGPSDELHDMTRRFWLGLLLAFPVLVLEMGSHLF   178
KO623silP       PEIRRSGPGSCPVCGMALEPLVATASTGPSDELHDMTRRFWLGLLLAFPVLVLEMGSHLF   178
                 *************************** ****:****************:********
```

b.

Multiple sequence alignment of silE (a.) and silP (b.) for Klebsiella oxytoca isolateed from wastewater The multiple equence alignment genearted using sequences of sil. Strictly conserved residues are makred with an asteriks (*). Conserved and semiconserved substitutions are represented by (:) and (.), dashes represent gaps. silE and silP identified using the database from the National Center for Biotechnology Information Protein. Ailgnment was performed using ClsutalW at http://custalw.genome.jp/