Whole genomes of trimethoprim resistant *Escherichia coli* isolates from urinary tract infections in New Zealand

Deanna Rozanne Athuraliya

A thesis submitted to Auckland University of Technology in the partial fulfilment of the requirements for the degree of Master of Science (Research) (MSc (Res))

2025

School of Science

Abstract

Urinary tract infections (UTIs) are one of the most common bacterial infections globally, affecting hospitals and communities with an estimated 150 million cases annually (Wagenlehner et al., 2020; Zagaglia et al., 2022). Infections are common with approximately 50% of females, 12% of men, and 5% of children experiencing a UTI episode in their lifetime (González et al., 2020). These infections are a large burden on affected individuals and healthcare systems due to the high morbidity and high medical costs involved, which are estimated to be more than $5 billion annually in the United States alone (Terlizzi et al., 2017).

Escherichia coli is a common facultative anaerobe in the mammalian gastrointestinal tract and can colonise the urinary tract as uropathogenic *E. coli* (UPEC) (Jaureguy et al., 2008; Kaper et al., 2004). This bacterium is responsible for approximately 90% of community-acquired UTIs (Flores-Mireles et al., 2015). Management of UTIs often uses empirical broad-spectrum antibiotics, making them the second most common cause for hospital antibiotic prescriptions (Pujades-Rodriguez et al., 2019; Zhou et al., 2023). The overprescription of antimicrobials may factor into the emergence of antimicrobial resistant (AMR) microorganisms such as *E. coli*. As it is one of the most critical AMR bacteria due to its ability to evade antibiotic treatment and transfer resistance genetic material to other species (Zhang et al., 2019).

Trimethoprim (TMP) is a synthetic folic acid antibiotic which was commonly used as a first-line treatment for UTIs and is now used in combination with sulfamethoxazole (SMX) as TMP-SMX. However, current global TMP prescription rates have declined due to the high resistance rates observed by recent clinical screenings (Schito et al., 2009; Zagaglia et al., 2022; Zhanel et al., 2006). While several resistance mechanisms have been identified in some key bacterial species throughout international research, the extent of clinical AMR in New Zealand (NZ) and resistance mechanisms are currently underexplored.

Antibiotic susceptibility testing (AST) was performed using disk diffusion assays and minimum inhibitory concentration (MIC) analysis to determine the resistance profiles of 106 *E. coli* isolates obtained from Middlemore Hospital (Auckland, NZ). Using disk diffusion assay AST, each isolate was tested against first-line antibiotics used to treat UTIs such as meropenem, TMP, TMP-SMX, amoxicillin-clavulanic acid, and nitrofurantoin, and the available national resistance data (BpacNZ, 2017; LabPLUS, 2023). One isolate was resistant to meropenem, 98 isolates were resistant to TMP, with 83 of these isolates being resistant to TMP-SMX. Eight isolates were resistant to amoxicillin-clavulanic acid, and two isolates were resistant to nitrofurantoin. Three of the 106 isolates were non-viable during culturing and were not investigated further. The 74

TMP-SMX resistant isolates underwent MIC testing where 24-hour optical density (OD) readings were generated at TMP concentrations ranging from 8-4096 µg/mL. These OD values were used to generate two criteria, based on growth rates features and estimated MIC, to classify isolates into low, medium, and high levels of TMP resistance. Based on these criteria, many of these isolates were classified as being of medium level resistance.

Results from MIC testing suggested resistant isolates were able to tolerate a TMP concentration of upwards of 128 times above the clinical breakpoint as demonstrated by OD curves. Therefore, whole genomes of six TMP-SMX resistant isolates were sequenced using Nanopore MinION (Oxford Nanopore Technology), with two isolates selected from each of the three TMP resistance levels generated from the two criteria. This aimed to identify differences in antibiotic resistance mechanisms within each resistant level. OrthoVenn3 and Comprehensive Antibiotic Resistance Database (CARD) were used to investigate novel resistance mechanisms, where efflux pumps and antibiotic target alterations were highlighted as potential mechanisms involved in *E. coli* TMP resistance, with the presence of the dihydrofolate reductase (*dfrA*) gene being an established mechanism (Brolund et al., 2010; Grape et al., 2007; Somorin et al., 2022). A total of 152 protein clusters were shared among all six isolates, with known AMR genes detected irrespective of the isolates TMP resistance, each containing two or three copies of the *dfrA* gene located on plasmids.

Overall, these results confirm the presence of TMP resistance in NZ *E. coli* isolates from UTIs. The level of TMP resistance shows no observable influence on the presence or absence of AMR genes. Future research on the expression levels of these genes to fully understand TMP resistance in UPEC.

Table of Contents

Abstract ii
List of Figures vii
List of Tables ix
Attestation of Authorship x
Acknowledgements xi
Abbreviations xiii
Chapter 1 General Introduction and Literature Review 1
1.1 General Introduction 1
1.2 Research Questions and Aims 2
1.3 Literature Review 6
1.3.1 Urinary Tract Infections 6
1.3.1.1 Uncomplicated Urinary Tract Infections 6
1.3.1.2 Complicated Urinary Tract Infections 7
1.3.1.3 Recurrent Urinary Tract Infections 8
1.3.2 Uropathogenic *Escherichia coli* 8
1.3.2.1 Morphology 10
1.3.2.2 Virulence Factors and Virulence 10
1.3.2.3 Genomic Characteristics 11
1.3.2.4 Natural Reservoirs 11
1.3.2.5 Growth in the Lab 12
1.3.3 Infection Diagnosis 12
1.3.3.1 Clinic and Practitioner Diagnosis 12
1.3.3.2 Laboratory Diagnostics 13
1.3.4 Antibiotic Therapeutic Treatment 13
1.3.4.1 Bacteriostatic Versus Bactericidal Antibiotics 15
1.3.4.2 Trimethoprim 15
1.3.4.3 Trimethoprim-Sulfamethoxazole 15
1.3.4.4 New Zealand Empirical Treatment 16
1.3.5 Antibiotic Resistance 18
1.3.5.1 Molecular Basis of Antibiotic Resistance 18
1.3.5.2 Uropathogenic *E. coli* Resistance and Trimethoprim Resistance 19
1.3.5.3 New Zealand Clinical Resistance Rates 22
1.3.5.4 Global Spread of UPEC Resistance 22
1.3.6 Antibiotic Susceptibility Testing 23
1.3.6.1 Disk Diffusion Assays 23
1.3.6.2 Broth Dilution Methods 24
1.3.7 Whole Genome Sequencing 25
1.3.7.1 Next Generation Sequencing 26
1.3.8 Bioinformatics and Comparative Genomics 27
1.4 Summary of Literature Review 27

Chapter 2 Antibiotic Susceptibility Testing of *E. coli* **28**
2.1 Introduction 28
2.2 Methods and Materials 33
2.2.1 Initial Testing and Acquisition of Trimethoprim Resistant Isolates 33
2.2.2 Confirmation of Antibiotic Susceptibility 33
2.2.3 Microbroth Minimum Inhibitory Concentration 35
2.2.3.1 Determination of Resistance Levels 35
2.2.3.2 Confirmation of Isolates 36
2.2.4 Ethics 37
2.3 Results 37
2.3.1 Confirmation of Antibiotic Susceptibility 37
2.3.2 Microbroth Minimum Inhibitory Concentration 39
2.3.2.1 Determination of Resistance Levels 39
2.3.2.2 Confirmation of Isolates 40
2.4 Discussion 44
2.4.1 Confirmation of Antibiotic Susceptibility 44
2.4.2 Determination of Resistance Levels and Confirmation of Isolates 46
2.4.3 Conclusion 48
Chapter 3 Whole Genome Sequencing of *E. coli* **49**
3.1 Introduction 49
3.2 Methods and Materials 54
3.2.1 Genomic DNA Extraction 54
3.2.1.1 Commercial Kit Extraction 54
3.2.2 Quality and Quantity of gDNA Extractions 54
3.2.3 AMpure XP Bead Purification 54
3.2.4 Oxford Nanopore Minion Library Preparation and Genome Sequencing 54
3.2.5 Genome Assembly 55
3.2.6 Whole Genome Analysis 55
3.2.7 Identification of Potential Novel Antibiotic Resistance Determinants 55
3.2.8 Investigation of *dfrA* Genes 56
3.3 Results 57
3.3.1 Genomic DNA Extraction 57
3.3.2 Whole Genome Analysis 57
3.3.3 Identification of Potential Novel Antibiotic Resistance Determinants 62
3.3.3.1 OrthoVenn3 62
3.3.3.2 The Comprehensive Antibiotic Resistance Database 65
3.3.3.3 Investigation of *dfrA* Genes 69
3.4 Discussion 73
3.4.1 Genomic DNA Extractions 73
3.4.2 Whole Genome Analysis 73
3.4.3 Identification of Potential Novel Antibiotic Resistance Determinants 73
3.4.4 Investigation of *dfrA* Genes 75
3.4.5 Novel Resistance Determinants and dfrA Genes Involvement in TMP Resistance 77
3.5 Conclusion 77

Chapter 4 Final Discussion ... 78
4.1 Antibiotic Susceptibility Testing of *E. coli* ... 78
4.2 Whole Genome Sequencing of *E. coli* ... 79
4.3 Future Directions ... 80
4.4 Conclusions ... 83
References ... 84
Appendices ... 111
Appendix A: Middlemore Hospital Antibiotic Susceptibility Testing ... 111
Appendix B: Confirmation of Antibiotic Susceptibility ... 112
Appendix C: Determination of Resistance Levels ... 112
Appendix D: OrthoVenn3 Gene Cluster Occurrence ... 112
Appendix E: OrthoVenn3 Resistance Cluster Biological Processes ... 112
Appendix F: OrthoVenn3 Resistance ... 112

List of Figures

Figure 1.1: Schematic of the RQs of this study. 5

Figure 1.2: Overview of infection caused by uropathogens (adapted from Fairweather et al. (2020) and Klein and Hultgren (2020)). 7

Figure 1.3: Main virulence factors of UPEC (retrieved from Totsika et al. (2012)).................... 10

Figure 1.4: Modes of action on UPEC cells by antibiotics used for UTI management (retrieved from Nasrollahian et al. (2024)).. 14

Figure 1.5: TMP-SMX points of action on the folate synthesis pathway (retrieved from Matos and Esteves (2010)).. 16

Figure 1.6: Percentage females dispensed nitrofurantoin or TMP for UTI treatment for the year ending December 2024 (retrieved from He Ako Hiringa (n.d)) 17

Figure 1.7: General resistance mechanisms against antibiotics in bacterial cells (retrieved from Darby et al. (2022)). 19

Figure 1.8: Overview of the THF metabolism encoded by the folA and folM genes in E. coli (adapted from Sangurdekar et al. (2011)). 20

Figure 1.9: Methods used for AST with the turnaround time (retrieved from Gajic et al. (2022)). PCR- polymerase chain reaction, qPCR- quantitative polymerase chain reaction, NGS- next generation sequencing, MALDI-TOF MS – matrix-assisted laser desorption/ionisation time-of-light mass spectrometry. 23

Figure 1.10: Interpretation of antibiotic breakpoint (retrieved from MacGowan and Wise (2001)).. 24

Figure 1.11: ONT MinION sequencing principle (retrieved from Wang et al. (2021))............... 26

Figure 2.1: Growth characteristics of an *E. coli* strain in the presence (red) and absence (black) of TMP (retrieved from Vinchhi et al. (2023)).. 29

Figure 2.2: Schematic of the RQs of this study. Chapter 2 RQs and aims are highlighted in red. 31

Figure 2.3: Chapter 2 method objectives.. 32

Figure 2.4: Schematic of disk layout on MHA during antibiotic disk diffusion AUT AST. 34

Figure 2.5: Schematic of 96-well plate layout during first round of microbroth MIC testing. ... 36

Figure 2.6: Schematic of 96-well plate layout during second round of MIC testing. 37

Figure 2.7: OD for triplicate 24-hour readings for the 12 isolates.. 41

Figure 2.8: Average total growth under curve for the 12 isolates. Blue indicates low TMP resistance, yellow indicates medium TMP resistance, and red indicates high TMP resistance. Clinical breakpoint of 32 µg/mL as stated by CLSI (2023) is shown by grey line. 43

Figure 2.9: Normalised OD for sterility wells for the 12 isolates.. 44

Figure 3.1: Schematic of the RQs of this study. Chapter 3 RQ and aims are highlighted in red. 52

Figure 3.2: Chapter 3 method objectives.. 53

Figure 3.3: Schematic of clusters of interest from generated OrthoVenn3 Venn diagram........ 56

Figure 3.4: 1% agarose gel of isolates gDNA using the Qiagen DNeasy Mini Blood and Tissue Kit. NTC = no template control used as a negative control.. 57

Figure 3.5: Assemblies of sequenced genomes as visualised by Bandage. 58

Figure 3.6: UpSet plot of orthologous clusters for isolates as predicted by OrthoVenn3. 63
Figure 3.7: Venn diagram of clusters in resistant isolates produced by OrthoVenn3. 65
Figure 3.8: Heatmap showing percentage similarity according to CARD of selected genes. 68
Figure 3.9: Nucleotide maximum likelihood phylogenetic tree of isolate *dfrA* variants. 71
Figure 3.10: Protein maximum likelihood phylogenetic tree of isolate *dfrA* variants. 72

List of Tables

Table 1.1: Empirical antibiotics for uncomplicated UTI in adults (adapted from BpacNZ (2021)). 17

Table 1.2: Mechanisms of intrinsic TMP resistance which have been investigated. 21

Table 1.3: Resistance results for *E. coli* isolates from adult and paediatric clinical specimens in 2022 (adapted from LabPLUS (2023)). 22

Table 2.1: Percentage of *E. coli* isolates susceptible to antibiotics. 29

Table 2.2: Zone diameter and interpretive categories for Enterobacterales as set in CLSI (2023). 34

Table 2.3: AST sensitivity results of isolates obtained from Middlemore Hospital. 38

Table 2.4: AST resistance results of isolates to drug classes. 38

Table 2.5: Isolate criterium for three resistance levels based on OD growth rate features. 39

Table 2.6: Isolate criterium for three resistance levels based on estimated MIC from growth curves. 40

Table 3.1: Examples of *dfrA* variant genes identified in *E. coli* TMP resistant isolates. 51

Table 3.2: BLASTn results of Bandage plasmids from each isolate. 59

Table 3.3: Genomic features of draft genomes of isolates from RAST. 61

Table 3.4: Antibiotic resistance mechanism CARD hits for each for each isolate. 67

Table 3.5: Presence of antifolate genes in isolates and the number of copies according to RAST. 69

Table 3.6: RAST and BLASTn results for *dfrA* genes from each isolate. 70

Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor used artificial intelligence tools or generative artificial intelligence tools (unless it is clearly stated and referenced, along with the purpose of use), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

Deanna Rozanne Athuraliya

Date: 14 September 2025

Acknowledgements

This is by far the easiest part of my thesis to write, as I have been so fortunate to have incredible support throughout my Masters. I share this accomplishment with you all, and to properly thank you all would need its very own chapter.

To my primary supervisor, Associate Professor Brent Seale, thank you for your encouragement, reassurance, and genuine care, which has guided me through both research and life. You have a passion for teaching and supporting your students. This is why we naturally find each other across industries and share so many fond memories of you. To my secondary supervisor, Dr Kevin Lee, thank you for sharing your wisdom on anything and everything statistics related. Your analogies for explaining molecular methods and sharing Sour Patch Kids during meetings will always stay with me. Thank you both for being part of Team UTI. I would also like to thank Dr Susan Taylor and the team at Middlemore Hospital for providing the *E. coli* isolates used in this thesis, without your help this project would not have been possible.

I would like to thank the staff at AUT, especially Professor Donnabella Lacap-Bugler, Associate Professor Colleen Higgins and the past and present members of the Microbiology and Molecular Microbiology Research Groups for all your support. Thank you for showing me what true passion and drive in science looks like. Thank you for challenging the way I think and articulate my research. To the wonderful ladies in the science labs especially Meie, Jinan, and Eileen, thank you for all your help and sharing your inspiring experiences as women in science, I hope I can do you ladies proud. Thank you also to Tim Lawrence for your molecular expertise during the genome sequencing portion of this research.

To my parents, thank you for emphasising the value of education and encouraging my love for science. From human anatomy Christmas gifts to late-night tutoring sessions and school science fair projects, you have always supported me throughout my education and encouraged my curiosity in biology. I look up to you in countless ways and owe my work ethic and my thirst for knowledge you both. Thank you for supporting me in everything I do and being proud of my accomplishments; words cannot thank you both enough. To my big brother Chrishane, naturally I have always looked up to you. Thank you for showing me what it means to step out of your comfort zone, for being the first to exceed other people's doubt, to not take no for an answer, and for being my first mentor in life. Your determination has taught me that anything is possible if you set your mind to it. You have shown me that just because you are afraid of heights, does not mean you should not aim for the skies. Thank you for showing me what true ambition and persistence looks like and encouraging me to have my head in the clouds. To Anas, thank you

for embracing my passion for research and sharing it proudly with your world, I am sure a few of your colleagues are now traumatised by antibiotic resistance. Thank you for the morning matcha deliveries, daily peptalks, proof reading, and evening pickups after long days of experiments and teaching. Whether five feet or 10 000 kilometres away, and everywhere in between, thank you for being steady and calm during my many overwhelmed moments. You have reminded me that my stubbornness is also my strength, and that I finish what I set my mind to. I could not have come this far without the fierce support from you four, thank you for always being my solid foundation, I love you all.

I would like to sincerely thank Zoe for all your guidance from day one. From late night data analysis video calls, proofreading drafts, to ranting about life, I genuinely could not have made it to this finish line without a friend like you. You have been my unofficial third supervisor and I cannot wait to see you cross your own research finish line soon. To my AUT friends, Amber, Laura, Marina, and Eliana, thank you for the chats in and around experiments and teaching, and showing me that girls in STEM can do anything. I am excited to see how you all shake up and contribute to your research realms.

To my large and loyal Massey University family, there are far too many of you to name, thank you for being so supportive as I embarked on my postgraduate journey away from our Palmerston North roots. To my Ferguson Street friends, Andie, Elise, and Ella, thank you for the countless chats, Wellness Walkies to physically pull me away from my computer, and endless cups of peppermint tea. Your loyalty will always be number one. To Ravindi, Liora, Ravindu, and the Kellers, thank you for always cheering me on in my accomplishments, and cheering me up when I needed encouragement. Whoever said you should not run away from your problems, obviously did not have friends like you all to run to, thank you.

To my extended family all over the world, thank you for your endless support since I was young. I may not be able to see you as often as we wish, but I always feel your love and prayers carrying me through my endeavours. To my lifelong friends especially Dewmi, Teresa, and Anchal, thank you for always encouraging my love of science, from when we were kids in school, to me in school as an adult. Thank you to my fluffy research assistants, Poncho and Tuna, for keeping me company through many late nights writing and reminding me that a nap, a snack, and a bit of sunbathing is all you need sometimes. I owe you both wet food.

Finally, I would like to thank the Microbiology team at Awanui Labs for their support during the final stages of my thesis. Thank you for being so understanding as I juggled full time work, full time thesis writing, and full-time exhaustion.

Abbreviations

%	Percentage
°C	Degree Celsius
kbp	Kilo base pair
µg/mL	Micro gram per mililitre
µL	Microlitre
mL	Millilitre
mm	Millimetre
mg	Milligram
nm	Nanometres
pH	Power of hydrogen
µg	Microgram
AMR	Antimicrobial resistance
AUC	Average total growth under the curve
AST	Antibiotic susceptibility testing
AUT	Auckland University of Technology
BID	Twice daily
BLAST	Basic local alignment search tool
CARD	Comprehensive Antibiotic Resistance Database
CDS	Coding deoxyribonucleic acid sequence
CFU	Colony forming units
CLSI	Clinical Laboratory Standards Institute
DHF	Dihydrofolic acid
DHPS	Dihydropteroate synthase
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
E-test	Epsilometer test
ESBL	Extended spectrum beta-lactamase
EUCAST	European Committee on Antimicrobial
gDNA	Genomic deoxyribonucleic acid
LB	Luria-Bertani broth
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MBC	Minimum bactericidal concentration
MEGA11	Molecular Evolutionary Genetics Analysis version 11
MDR	Multi drug resistance
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth
MIC	Minimum inhibitory concentration

NCBI National Center for Biotechnology Information

NGS Next generation sequencing

nt Nucleotide

NTC No template control

NZ New Zealand

OD Optical density

ONT Oxford Nanopore Technology

OPC Orthologous protein cluster

QID Once a day

RAST Rapid Annotations using Subsystems Technology

RNA Ribonucleic acid

RQ Research question

SEED Shared Genome Database

SMX Sulfamethoxazole

spp. Species

THF Tetrahydrofolate

tRNA Transfer ribonucleic acid

TMP Trimethoprim

TMP-SMX Trimethoprim- sulfamethoxazole

UPEC Uropathogenic *Escherichia coli*

Chapter 1 General Introduction and Literature Review

1.1 General Introduction

Antimicrobial resistance (AMR) is a growing global public health crisis, contributing to increased morbidity and mortality. Nearly all human associated pathogens exhibit AMR, burdening healthcare systems with over 700,000 deaths from drug-resistant infections annually (Melnyk et al., 2015; Zhang et al., 2019). Currently, antibiotics target several proteins in bacteria where genetic mutations of these targets can result in widespread resistance to multiple antibiotic classes (Manna et al., 2021; Tamer et al., 2019). This concern is increased when resistant bacteria transfer mobile genetic elements and other microbial characteristics which enhance resistance (Pattis et al., 2022; Sheikh et al., 2019).

Urinary tract infections (UTIs) are the second most common clinical infection for antibiotic treatment in primary and secondary care, with catheter-associated UTIs being the most common nosocomial infection (Chen et al., 2019). Inaccurate patient diagnosis and treatment of UTIs may result in serious outcomes, including treatment failure, diagnostic errors, antibiotic related adverse reactions, and promoting antibiotic resistance (Chen et al., 2019). The antibiotic trimethoprim (TMP) was introduced in the 1960s as a first line treatment for uncomplicated UTIs in adults. This antibiotic was primarily used to treat uropathogenic *Escherichia coli* (UPEC), which accounts for approximately 90% of community-acquired UTIs (Flores-Mireles et al., 2015). However, its efficacy is now limited due to the rapid increase of TMP resistance globally (Flores-Mireles et al., 2015; D. S. Lee et al., 2018b; Manna et al., 2021). As a result, major international and New Zealand (NZ) guidelines now recommend short-term courses for oral antibiotics for UTI management and addressing antibiotic stewardship (BpacNZ, 2017; Novelli & Rosi, 2017).

The development of new antibiotics may be insufficient in combating the AMR public health crisis, rather an understanding of the mechanisms driving resistance, alongside antibiotic stewardship and community education is the ideal strategy (Lewnard et al., 2024; McCormick et al., 2020; Santana et al., 2024). Resistance to TMP in *E. coli* isolated from urine samples has been established by the presence of dihydrofolate reductase (*dfrA*) genes (Brolund et al., 2010; Grape et al., 2007; Somorin et al., 2022). There is little understanding about infection cycles in humans, especially mechanisms of antifolate resistance associated with gram-negative clinical samples (Shi et al., 2021; Toulouse et al., 2020). Understanding the mechanisms involved in *E. coli* antibiotic resistance is crucial for explaining treatment failure whilst predicting which treatments may soon become ineffective (Santana et al., 2024). While UPEC pathogenesis has been widely studied internationally, much of this research relies on computer simulations or

murine models to understand common evolutionary resistance caused by target mutations (Birkegård et al., 2018; Mulvey et al., 2000). However, these resistance strategies have not been fully established at biological and molecular levels during the treatment of uncomplicated UTIs (D. S. Lee et al., 2018b; Manna et al., 2021; Mulvey et al., 2000; Subashchandrabose et al., 2014; Tamer et al., 2019).

Published data on AMR bacteria and mechanisms of resistance in NZ is limited and outdated (Pattis et al., 2022). Furthermore, studies on clinical *E. coli* infections and antibiotic resistance are limited, with no investigations into their underlying molecular mechanisms of resistance (Heffernan et al., 2009; LabPLUS, 2023; Mangin et al., 2005; Pattis et al., 2022; Williamson & Heffernan, 2014). With a few whole genomes of NZ UPEC isolates currently available, a larger investigation of resistance mechanisms using whole genome sequencing (WGS) is needed to understanding AMR.

The increase in global AMR is a clinical challenge which greatly impacts individuals and healthcare resources (Ahmed et al., 2024). The high occurrence of resistant *E. coli* in NZ, along with the reduced effectiveness of TMP driven by rising global AMR rates, highlights the importance of studying this pathogen. To understand why antibiotic treatment is failing, it is important to investigate antibiotics which have already failed and therefore create better guidelines around antibiotic stewardship for the treatment of UTIs. The purpose of this thesis is to contribute to knowledge focused on antibiotic resistance mechanisms by *E. coli* in NZ using AST, WGS, and comparative genomic analysis. The aims of this project are listed below in Section 1.2 and presented inFigure 1.1.

1.2 Research Questions and Aims

Research question (RQ) 1: What antibiotics are UPEC isolates resistant to and at what concentrations?

> **Aim 1.1:** To confirm isolates obtained from Middlemore Hospital phenotypically demonstrate TMP resistance.
>
> > **Objective 1.1.1:** Disk diffusion assays using different antibiotic disks will be placed onto agar containing different UPEC isolates. Zones of inhibition will be measured and recorded. Isolates which are susceptible to TMP containing disks will be used for further testing.
>
> **Aim 1.2:** To determine what antibiotics UPEC isolates are resistant to.

Objective 1.2.1: Disk diffusion assays using different antibiotic disks will be placed onto agar containing different UPEC isolates. Zones of inhibition will be measured and recorded.

RQ2: How does the growth rate vary between TMP resistant UPEC isolates (determined by RQ1) in the presence of varying concentrations of TMP at 37°C?

Aim 2.1: To determine a criterium for low, medium, and high levels of TMP resistance.

Objective 2.1.1: Minimum inhibitory concentration (MIC) testing will be carried out using a 96-well plate and two-fold increasing TMP concentrations of 8 - 4096 µg/mL, where 24-hour absorbance readings will be taken.

Objective 2.1.2: Criteriums will be generated based on OD features and estimated MIC. Isolates will be organised within low, medium, and high levels of TMP resistance based on these criteria.

Aim 2.2: To interpret growth rates of isolates between low, medium, and high TMP resistance levels.

Objective 2.2.1: Isolates within each TMP resistance level will be analysed based on growth rate features such as steepness of curves and MIC.

Objective 2.2.2: Isolates from each of the TMP resistance levels will be chosen for further testing.

RQ3: What are the genetic differences of the whole genomes of differing levels of TMP resistant UPEC strains in the absence of the antibiotic?

Aim 3.1: To assemble the whole genomes of isolates at each of the three resistance levels.

Objective 3.1.1: After Oxford Nanopore MinION sequencing, whole genomes will be constructed using *de novo* assembly.

Objective 3.1.2: The quality of the constructed genomes will be visualised using Bandage and annotated using Rapid Annotations using Subsystems Technology (RAST).

Aim 3.2: To investigate the genomic variations between sequenced genomes.

Objective 3.2.1: Orthologous protein clusters will be identified and compared among whole genomes and gene sets of isolates from Aim 3.1 using OrthoVenn3.

Objective 3.2.2: The Comprehensive Antibiotic Resistance Database (CARD) will be used to identify known antibiotic resistance genes present in isolate genomes from Aim 3.1.

Objective 3.2.3: Identified genes associated with antibiotic resistance will be compared among isolates to identify similar resistance gene mechanisms between low, medium, and high TMP resistance levels.

Objective 3.2.4: Phylogenetic trees of the nucleotide and protein sequences from *dfrA* genes of each isolate will be generated using MEGA11.

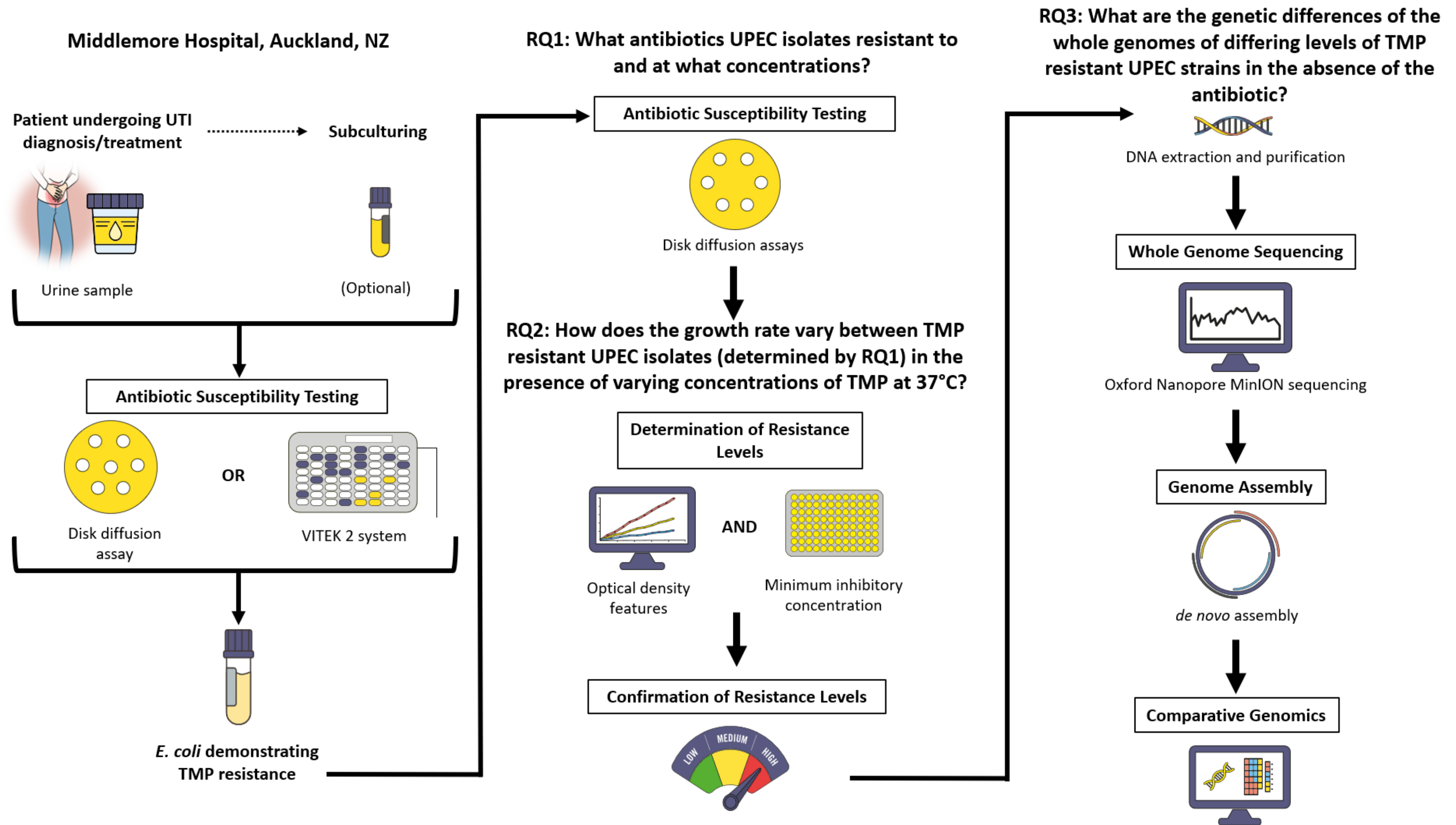

Figure 1.1: Schematic of the RQs of this study.

1.3 Literature Review

1.3.1 Urinary Tract Infections

Urinary tract infections (UTIs) are one of the most common global bacterial infections in communities and healthcare systems with approximately 150 million individuals affected annually (Chagneau et al., 2021; Wagenlehner et al., 2020; Zagaglia et al., 2022). At least 40% of women, 12% of men, and 5% of children will experience a UTI episode in their lifetime (González et al., 2020). Adult women have the highest rates of infection with 50-60% (Medina & Castillo-Pino, 2019). This is a large concern for affected individuals and their supporting healthcare systems.

Classifications of UTIs vary depending on the risk factors, clinical presentation (i.e., symptomatic, or asymptomatic), and the acquired source of the infection. Infections may be acquired through hospitals, wider healthcare systems, or within communities (Arafi et al., 2023). The urinary tract is a sterile environment, excluding the distal urethra (Sarowska et al., 2019). Therefore, individuals with structural or functional urinary tract abnormalities have a higher risk for UTI occurrence (D. S. Lee et al., 2018b). This determines the treatment plan for the individual (Arafi et al., 2023).

1.3.1.1 Uncomplicated Urinary Tract Infections

Individuals with uncomplicated UTIs typically lack urinary tract abnormalities, catheterisation, or obstruction, and their healthy immune systems are usually able to control the infection (Arafi et al., 2023). For example, uncomplicated UTIs may be seen in 20-80% of healthy non-pregnant females with asymptomatic bacteriuria (≥ 10^5 CFU/mL bacteria in urine without clinical symptoms) as their immune systems are able to resolve the infection and treatment is not required (Schneeberger et al., 2014).

The progression of infection by pathogens (uropathogens) into the urinary tract may affect other organs within the urinary system (Figure 1.2). Urethritis is used to describe the presence of uropathogens in the ureter where urine may contain bacteria (bacteriuria) or neutrophils and pus (pyuria) (Ghosh et al., 2021; Luna-Pineda et al., 2016; Ussher et al., 2020). When the infection progresses to the bladder it is known as cystitis, which is prevalent in females with approximately 11% over the age of 18 years developing annual cystitis infections (Flores-Mireles et al., 2015; Silverman et al., 2013; Terlizzi et al., 2017). Lower UTIs in males may cause pyelonephritis or genital infections such as prostatitis and epididymo-orchitis, with the potential to progress to urosepsis (Wagenlehner et al., 2020). Lower UTIs involve the lower organs of the

urinary system and are classified as being 'uncomplicated' as they are typically benign and have not progressed to the kidneys or into the bloodstream (Klein & Hultgren, 2020; Wagenlehner et al., 2020).

1.3.1.2 Complicated Urinary Tract Infections

Complicated UTIs are often associated with urinary catheterisation and obstruction or long urine retention (Hong et al., 2020). These increases UTI persistence, increase the risk of recurrent infections, and may require longer treatment therapies (Arafi et al., 2023).

Upper UTIs are classified as being 'complicated' as they may have progressed to the kidneys or into the bloodstream (bacteraemia) resulting in urosepsis and becoming life-threatening (Klein & Hultgren, 2020; Wagenlehner et al., 2020). This may be the result of infection and pus within the kidneys causing obstruction and resulting in pyonephrosis due to uncomplicated UTIs not being treated effectively (Ghosh et al., 2021; Ussher et al., 2020; J. Yang et al., 2021).

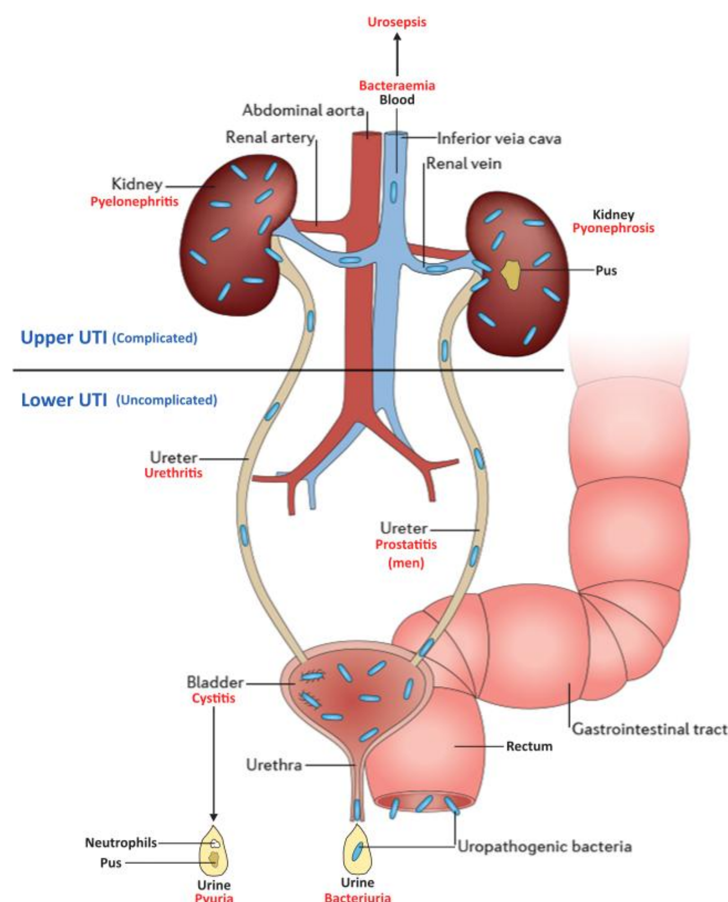

Figure 1.2: Overview of infection caused by uropathogens (adapted from Fairweather et al. (2020) and Klein and Hultgren (2020)).

1.3.1.3 Recurrent Urinary Tract Infections

Along with being common infections, UTIs also have a high recurrence rate (Zhou et al., 2023). Recurrent UTIs are defined by two confirmed episodes within a six-month period, or three episodes within a 12-month period (Churchill, 2023; Murray et al., 2021). Recurrence is most common in adult females, where more than 25% of females experience an infection within three to four months of the initial infection (Churchill, 2023; Kao et al., 2023; Mann et al., 2017; Murray et al., 2021; Terlizzi et al., 2017). Uropathogenic strains causing recurrent UTIs may be the same or different from alternative origins such as from the bladder epithelium or by strains commonly found in the gut which are then able to re-inoculate the bladder (Johnson & Russo, 2005).

1.3.2 Uropathogenic *Escherichia coli*

Different pathogenic organisms, including gram-positive and gram-negative bacteria and fungi, can cause UTIs (Ruța et al., 2024). Uropathogenic bacteria are the primary causative organisms of UTIs as they typically reside in the intestine and can infect the periurethral region. They are then able to colonise the urethra and spread towards the bladder (Flores-Mireles et al., 2015). Uropathogenic bacterial species varying between the different UTI types such as uncomplicated and complicated cystitis, pyelonephritis, and urosepsis (Wagenlehner et al., 2020). Uropathogenic *Escherichia coli* (UPEC) is the leading pathogenic cause of UTIs, accounting for more than 80% of reported cases (Flores-Mireles et al., 2015; Wagenlehner et al., 2020). Other bacterial species such as *Klebsiella* spp., *Staphylococcus* spp., *Enterobacter* spp., *Proteus* spp., and *Enterococcus* spp., may also cause UTIs to a lesser extent than UPEC (Flores-Mireles et al., 2015; Foxman, 2014; Wagenlehner et al., 2020).

Escherichia coli is a non-sporulating gram-negative rod-shaped bacterium (Rozwadowski & Gawel, 2022; Ye et al., 2022). *E. coli* is classified into one of three groups: commensal (non-pathogenic) strains which exist with the host without causing disease, intestinal pathogenic (diarrheagenic) strains, or extraintestinal pathogenic strains (Lloyd et al., 2009). It typically colonises the mucosal layer in mammalian colons and is the most abundantly found facultative anaerobe as part of the human gastrointestinal tract flora (Jaureguy et al., 2008; Kaper et al., 2004). Colonisation of the human gastrointestinal tract begins within the first few hours of birth. This microbial community synergistically supports digestions, synthesising essential vitamins, maintaining microbial balance, and regulating physical and chemical environments of the human gut (Etefia & Solomon, 2020; Kaper et al., 2004; Sarowska et al., 2019). *E. coli* can reside in the urinary microbiome without causing irritation, as many strains are non-pathogenic as part of the normal human flora (Abdelwahed et al., 2022; Crum et al., 2023). They are opportunistic strains where they rarely cause disease unless the host is immunocompromised or gastrointestinal

barriers are disturbed (Braz et al., 2020; Kaper et al., 2004). UPEC is one of the few uropathogens causative of both uncomplicated and complicated UTIs (Flores-Mireles et al., 2015). Studies have shown that UTI patients often have high concentrations of UPEC strains in their gut, which is predicted to increase the risk of recurrent infections (Nielsen et al., 2014; Yamamoto et al., 1997). UPEC strains can cause a large excessive host inflammation response which causes uroepithelial apoptosis that impairs innate immune cell function by exposing deeper immature cells to UPEC (González et al., 2020; Wu et al., 2022). These mechanisms are often seen in UPEC associated recurrent UTIs, as nearly half of recurrent uncomplicated cystitis cases being caused by the same strain as the initial infection (Murray et al., 2021; Rosen et al., 2007). This persistence places a considerable burden on the suffering patients, healthcare systems, and the economy (Flores-Mireles et al., 2015).

Uroepithelial cells are typically the first to respond to microbial invasion, coordinating subsequent host responses through the release of cytokines, inflammation, complement proteins, and the influx of neutrophils. When UPEC infects uroepithelial cells, toxins such as haemolysis and cytotoxic necrotising factor-1 are produced to mediate direct cell lysis or injury, exposing the mucosal barrier, and allowing the access to the underlying tissues. With the exposed urinary tract mucosa, the established UPEC triggers rapid host immune responses (Wu et al., 2022). UPEC pathogenesis involves colonisation of the periurethral area and urethra via fimbria where UPEC cells ascend via the bladder lumen, allowing planktonic cells to flourish and be shed in the urine. Cells adhere to the bladder epithelium triggering host cells defence systems and initiating apoptosis, allowing bladder invasion and replication results in the formation of bacterial communities of intracellular biofilm like structures (Flores-Mireles et al., 2015; Silverman et al., 2013; Terlizzi et al., 2017). UPEC forms protective structures that promote reinfection in the urothelial cytoplasm by causing bladder cell lysis (Luna-Pineda et al., 2016; Martínez-Figueroa et al., 2020). Exfoliated epithelial cells are then excreted in urine while intracellular reservoirs may be present in a dormant state which allows for long term survival and reinfection (Luna-Pineda et al., 2016; Rosen et al., 2007). Reactivation occurs via exocytosis where UPEC can reinfect new superficial epithelial cells and initiate another infection cycle (González et al., 2020; Luna-Pineda et al., 2016). Kidney colonisation by UPEC occurs via adhesions or pili which damage host tissue by producing toxins, and allowing UPEC to penetrate the epithelial barrier which can result in bacteraemia and septicaemia (Flores-Mireles et al., 2015; Silverman et al., 2013; Terlizzi et al., 2017).

1.3.2.1 Morphology

There are morphological variations by UPEC during infection, whereby they typically exist as short rods or nearly spherical cells within biofilm communities (Justice et al., 2008). After host cells have been lysed, dispersed subpopulations may appear as rods, motile rods, or filamentous (Justice et al., 2006). This filamentation is caused by elongation and chromosomal replication in the absence of environmental stress, and is characteristic of UPEC in UTIs as it promotes dispersal, attachment, and phagocytosis evasion (Arafi et al., 2023; Horvath et al., 2011; Justice et al., 2006; Möller et al., 2013).

1.3.2.2 Virulence Factors and Virulence

Intestinal *E. coli* strains originating from the gastrointestinal tract may differ in growth factors and persistence at infection sites, therefore they result in diverse virulence profiles when they colonies the urinary tract (Geurtsen et al., 2022). Virulence factors in UPEC are adhesions including fimbriae and pili, iron acquisition systems, toxins, protective surface structures such as a capsule, protective surface structures, biofilm formation, and immune evasion proteins Figure 1.3 (Murray et al., 2021; D. Yang et al., 2021).

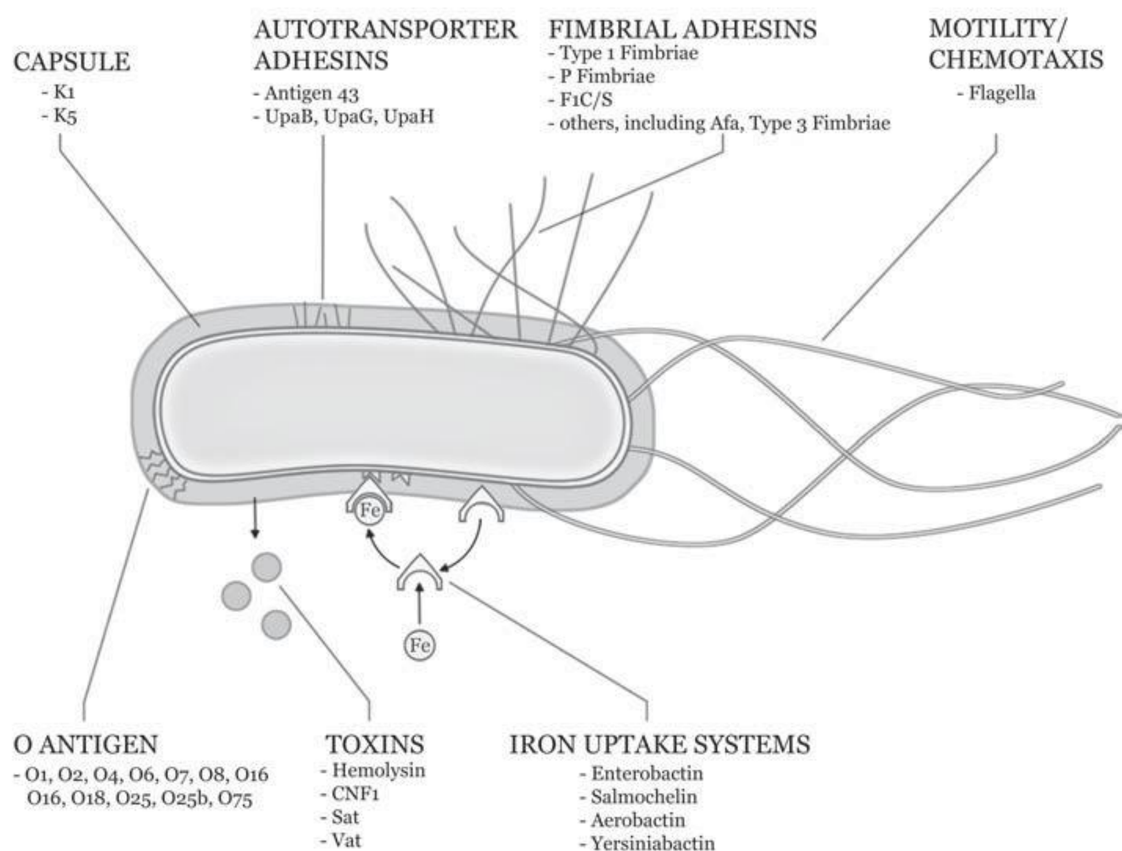

Figure 1.3: Main virulence factors of UPEC (retrieved from Totsika et al. (2012)).

These factors allow adherence, invasion, toxicity, effector molecule delivery, and motility (Croxen & Finlay, 2010; Kaper et al., 2004). They contribute to immune evasion by overcoming antigenic recognition, evading phagocytosis, counteracting proteolysis, whilst enhancing stress tolerance, promoting essential ion uptake such as iron and magnesium, and regulating other virulence determinants (Geurtsen et al., 2022; Terlizzi et al., 2017).

UPEC virulence factors allow for the survival in low oxygen and nutrient environments such as the bladder and kidney by allowing adherence, colonisation, host cell invasion, tissue damage, and immune evasion in the host (Murray et al., 2021; D. Yang et al., 2021). Biofilm formation also contributes to UPEC virulence and contributes to pyelonephritis infection and the development of chronic or recurrent UTIs (Soto et al., 2006).

1.3.2.3 Genomic Characteristics

Pathogenic *E. coli* strains generally have larger genomes than commensal strains, mainly due to the acquisition of large chromosomal insertions (Lloyd et al., 2009; Rode et al., 1999). As a result, genome sizes vary in length from approximately 4.7 – 5.1 kilo base pairs (kbp) (Rode et al., 1999). Studies investigating UPEC strains have reported larger genome sizes with approximately 5.3 kbp and an average guanine and cytosine content of 51% (Lloyd et al., 2009; Subhadra et al., 2018). As sequencing technology advances, more *E. coli* isolates are analysed, increasing the number of gene families which are identified (Rouli et al., 2015). Studies have identified approximately between 1000 and 3000 genes in *E. coli* genomes using technology such as microarrays and sequencing (Geurtsen et al., 2022). Many of these genes encode essential housekeeping proteins which are involved in replications, transcription, translation, and necessary basic metabolic functions (Leimbach et al., 2013; Rasko et al., 2008). The genes which are encoded include those involved in transport processes such as efflux pumps (Teelucksingh et al., 2020). This gene variation in *E. coli* genomes may allow for adaptive advantages when in competitive microbial communities with other bacterial species (Geurtsen et al., 2022). Commensal *E. coli* strains can act as genetic repositories by acquiring and transferring deoxyribonucleic acid (DNA) from other strains and contributing to the emergence of pathogenic isolates (Rasko et al., 2008). Conversely, pathogenic strains may revert to commensalism by losing or transferring genetic material such as via plasmids (Geurtsen et al., 2022).

1.3.2.4 Natural Reservoirs

The human gastrointestinal tract is the primary natural reservoir of *E. coli*, and rarely causes complications in healthy individuals (García et al., 2023). Approximately half of complicated UTI cases originate from commensal *E. coli* strains, while the remaining half are caused by

extraintestinal pathogenic *E. coli* (Chen et al., 2020). This colonisation of the enteric rectal flora resulting in UTIs such as cystitis has been recognised as the faecal-perineal-urethral hypothesis (Yamamoto et al., 1997). Recent studies have shown that patients with recurrent UTIs are often reinfected by uropathogenic strains which reproduce within the intestinal reservoir (Johnson & Russo, 2005; Kaper et al., 2004; Thänert et al., 2019; Ukah et al., 2018). This has been supported through several studies where faecal samples from patients presenting symptomatic UTIs exhibit the same strain of UPEC in their stool (Thänert et al., 2019). Further studies have suggested that intestinal *E. coli* present in the intestinal microbiota is able to exchange virulence factors between cells, promoting the emergence of hybrid intestinal *E. coli* which are pathogenic and result in UTIs (Gati et al., 2019; Lara et al., 2017; Santos et al., 2020; Valiatti et al., 2020).

1.3.2.5 Growth in the Lab

Bacterial growth and the growth rate refer to the increase in cell number or biomass over time under controlled conditions (Wiegand et al., 2008). Bacteria are cultivated on agar plates or in liquid broth to investigate phenotypes and novel bacterial functions (Boolchandani et al., 2019). *E. coli* can grow in minimal media containing carbon sources such as glucose with nitrogen, phosphorus, and trace metals present (Elbing & Brent, 2019). In the laboratory, *E. coli* has a doubling time of approximately 20 minutes in media with optimal pH and nutrients and incubated at 37°C and aerobic conditions (Tuttle et al., 2021). Growth is faster in rich media where amino acids and other metabolites are available to support cell synthesis (Elbing & Brent, 2019). Studies have used Luria Bertani broth (LB) and artificial urine to compare the nutrients available to cell growth as virulence gene expressions may vary between the two media types and certain strains may prefer the differing nutrients (Koudounas et al., 2021; Sarshar et al., 2022).

1.3.3 Infection Diagnosis

Diagnosis is typically performed in three stages. First, during triage where practitioners assess patients through targeted questions to determine whether testing is needed and which tests to perform. Second, a sample from the patient is analysed using laboratory-based methods. Third, the test results are communicated back to the patient (Versluis et al., 2022).

1.3.3.1 Clinic and Practitioner Diagnosis

Clinical diagnosis of UTIs is commonly characterised by patient symptoms such as pelvic pain, urination frequency and urgency, nausea, or fever (Bilsen et al., 2023). Pelvic discomfort is a noticeable symptom experienced by individuals as urinary tract pathogens initiate epithelial inflammation (Ussher et al., 2020; Warren et al., 2011). However, some patients may be

asymptomatic without the typical symptoms of dysuria or frequency but have significant urinary tract bacterial colonisation (Nicolle et al., 2019).

The dipstick method is one of the most commonly used diagnostic test for UTIs in healthcare settings, as it detects infection indicators in urine (Bilsen et al., 2023; Dadzie et al., 2019; Somorin et al., 2022). A positive dipstick test is where high red blood cells, neutrophils and nitrates in patient urine is identified and therefore suggests an infection (Dadzie et al., 2019). These are indicators as they reflect the host immune responses, such as high production of neutrophils directed to infected bladder cells and the production of nitric oxide and defensins by urothelial cells (Mulvey et al., 2000). While the dipstick test is inexpensive and rapid, it provides limited information as not all patients with bacteriuria display UTI symptoms (asymptomatic UTIs) and its accuracy varies across populations and urine outputs (Bafna et al., 2020; Lammers et al., 2001; Marques et al., 2017). Clinical evidence suggests variability in pathogen cell counts in urine between symptomatic patient cases (Churchill, 2023; Wagenlehner et al., 2020). Therefore, laboratory-based diagnostics are recommended to ensure the most appropriate treatment plan is made.

1.3.3.2 Laboratory Diagnostics

Laboratory diagnostic testing refers to the testing to determine the presence of a disease through the culturing and analysis of patient urine to determine the most suitable treatment plan (Bafna et al., 2020; Versluis et al., 2022). Laboratory diagnostic methods may include microscopy and flow cytometry to quantify pyuria, as well as blood and urine culturing and susceptibility testing (Bilsen et al., 2023). The presence of over 10^5 colony forming units of a pathogen per millilitre (CFU/mL) of urine in a midstream urine sample has been used as an indication of infection during urinalysis (Al Lawati et al., 2024). However, studies have suggested that lower CFU/mL may be clinically relevant especially in symptomatic patients (Churchill, 2023; Terlizzi et al., 2017; Zhou et al., 2023).

1.3.4 Antibiotic Therapeutic Treatment

Patients with UTIs are often diagnosed in clinics by a positive dipstick as described in Section 1.3.3.1. This guides empirical treatment which is often managed with antibiotic therapy (Dadzie et al., 2019; Zhou et al., 2023). UTIs are the second most common use of nosocomial antibiotic prescriptions globally where broad spectrum agents widely prescribed (Sharma et al., 2021; Zhou et al., 2023). Some first line antibiotic recommendations for these infections include nitrofurantoin, trimethoprim-sulfamethoxazole, pivmecillin, and fosfomycin (Luna-Pineda et al., 2016; Zhou et al., 2023).

Antibiotics act through five main mechanisms of action: blocking bacterial cell wall synthesis, disrupting protein biosynthesis, interfering with nucleic acid replication, impairing bacterial metabolic pathways, or inhibiting membrane function Figure 1.4 (Kapoor et al., 2017).

Bacterial cell walls are supported by peptidoglycan, making them an antibiotic target by beta-lactams (β-lactams) and fosfomycin to disrupt peptidoglycan synthesis while glycopeptides inhibit chain elongation which leaves bacterial cells vulnerable to lysis (Bush & Bradford, 2016; Uddin et al., 2021; Wang et al., 2018). Protein biosynthesis is inhibited in ribosomes by aminoglycosides and tetracyclines targeting the 30S subunit and macrolides, chloramphenicol, and oxazolidinones targeting the 50S subunit (Krause et al., 2016). Fluroquinolones interfere with topoisomerase to block DNA replication, while nitrofurantoin damages bacterial DNA directly (Raheem & Straus, 2019). Antifolates may act synergistically to block important enzymes in the folate pathway which inhibits bacterial nucleotide synthesis (Fernández-Villa et al., 2019). Membrane targeting agents act to compromise cell integrity which increases susceptibility to osmotic stress and antibiotic action (Lohner & Blondelle, 2005).

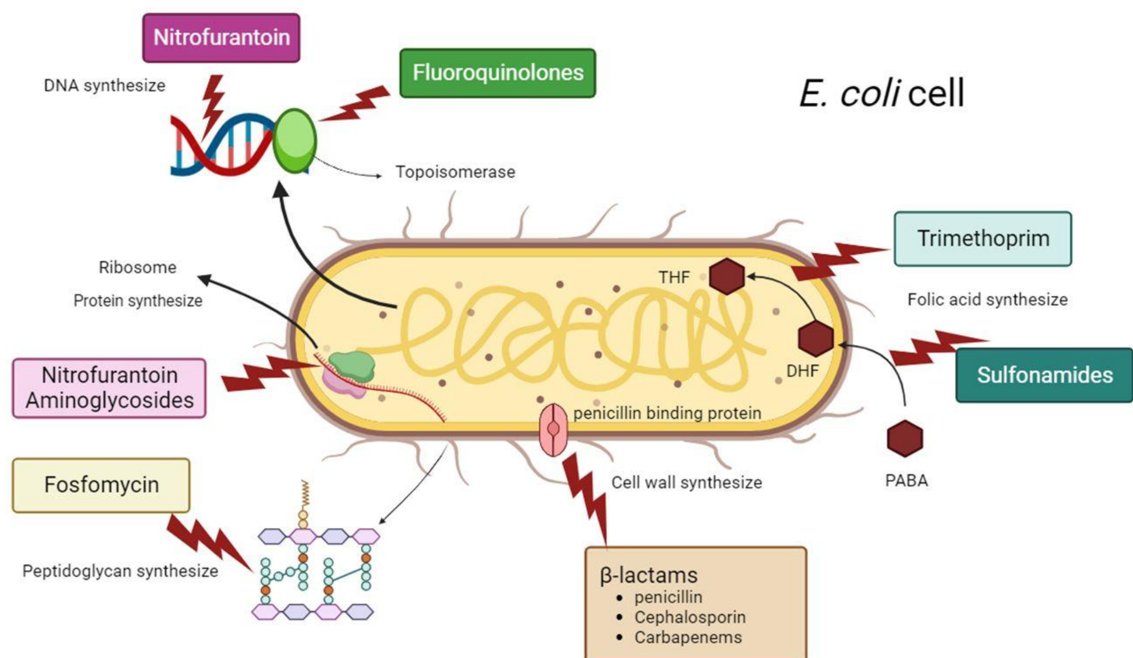

Figure 1.4: Modes of action on UPEC cells by antibiotics used for UTI management (retrieved from Nasrollahian et al. (2024)).

1.3.4.1 Bacteriostatic Versus Bactericidal Antibiotics

Antibiotics which kill bacteria are classified as bactericidal while those that inhibit bacterial growth are bacteriostatic and rely on host immune responses, primarily phagocytic clearance, to eliminate infection (Nemeth et al., 2014). Clinically, bactericidal agents are preferred for treating severe infections, especially in patients who are immunocompromised or with conditions such as endocarditis (Habib et al., 2009; Nemeth et al., 2014).

1.3.4.2 Trimethoprim

Folic acid or folate typically refers to tetrahydrofolate (THF) which is a cofactor involved in metabolic functions which are essential for cell survival (Shi et al., 2021; Wüthrich et al., 2019). Folate metabolism is involved in multiple biosynthesis pathways, including the synthesis of amino acids such as methionine, serine, glycine, histidine, and glutamate, as wells as purine and pyrimidine bases required for DNA and RNA (Brosnan et al., 2015). Folic acid also contributes to the regulation of gene expression through one carbon transfer reactions (Fernández-Villa et al., 2019).

Folate antagonists have been developed to target bacterial dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR), which are enzymes essential for folate synthesis and metabolism (Shi et al., 2021; Wüthrich et al., 2019). Trimethoprim (TMP) is a bacteriostatic synthetic folic acid antagonist introduced in the 1960s and is commonly administered in the combination with the sulfonamide antibiotic sulfamethizole (SMX) as trimethoprim-sulfamethoxazole (TMP-SMX) or co-trimoxazole (Fernández-Villa et al., 2019; Shi et al., 2021). Inhibiting DHFR disrupts folate metabolism resulting in impaired thymidine biosynthesis, disruptions in DNA replication, and eventually bacterial cell death (Schober et al., 2019).The World Health Organisation has included TMP as an important antibiotic for global health (World Health Organisation, 2024). TMP selectively inhibits bacterial DHFR which blocks the reduction of dihydrofolic acid (DHF) to THF (Diaz-Diaz et al., 2022; Kordus & Baughn, 2019; Lemay-St-Denis et al., 2021; Tamer et al., 2019; Toulouse et al., 2020). Therefore, the selective inhibition of DHFR is effective as THF is vital for protein and nucleic acid biosynthesis (Adrian & Klugman, 1997; Lauxen et al., 2021).

1.3.4.3 Trimethoprim-Sulfamethoxazole

The combination of TMP-SMX is widely used as empirical therapy, particularly in immunocompromised individuals due to its synergistic bactericidal effect and reduced toxicity at lower dosages (Diaz-Diaz et al., 2022; Kordus & Baughn, 2019; Wrobel et al., 2020). While TMP alone is bacteriostatic when combined with SMX they are bactericidal (Nakamura et al.,

2025). TMP contributes not only by inhibiting DHFR but also promoting reactive oxygen under aerobic conditions which triggers redox stress and secondary cellular damage (Diaz-Diaz et al., 2022; Kordus & Baughn, 2019). Together, TMP-SMX sequentially inhibits bacterial THF synthesis, and two enzymatic steps as shown in Figure 1.5. This blocks thymidine and purine production, which are essential for DNA and RNA biosynthesis (Wüthrich et al., 2019).

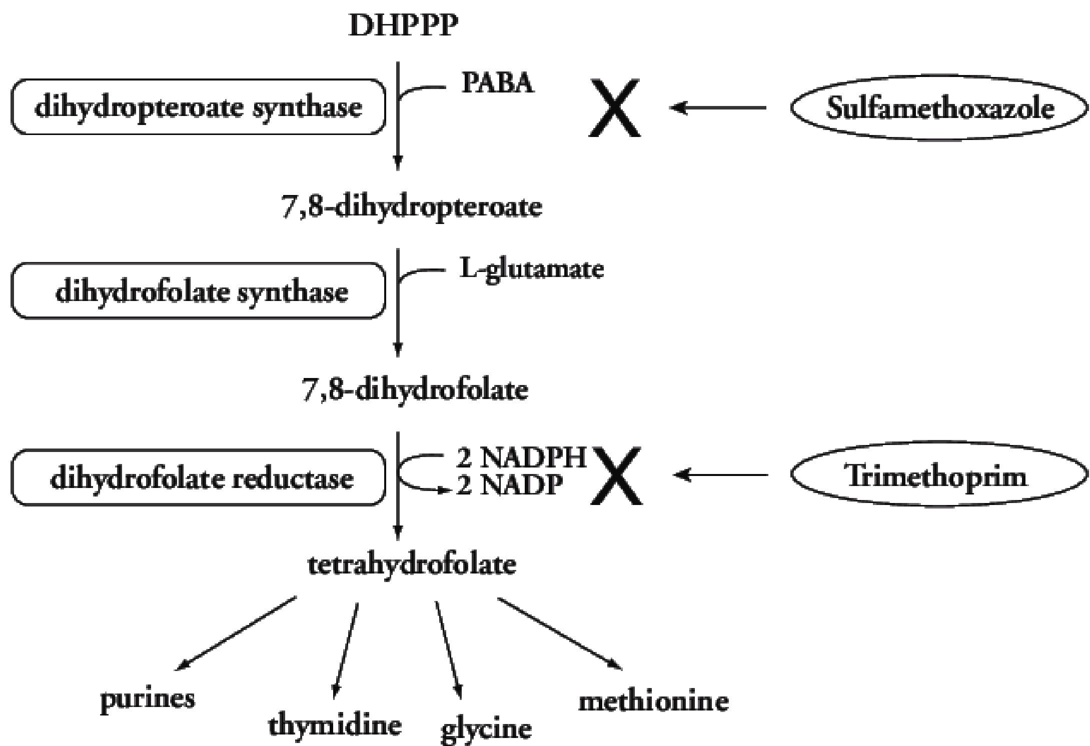

Figure 1.5: TMP-SMX points of action on the folate synthesis pathway (retrieved from Matos and Esteves (2010)).

1.3.4.4 New Zealand Empirical Treatment

Management of UTIs in New Zealand (NZ) is done typically through empirical antibiotic therapy where prescriptions are given at the time of diagnosis without urine culturing and confirmation of the causative pathogen (Carmona-Cartaya et al., 2022). As pathogen identity and resistance profiles are unknown without culturing, treatment decisions are guided by clinician judgement and local epidemiological antibiotic susceptibility data (Pujades-Rodriguez et al., 2019). As shown in Table 1.1, only a limited number of antibiotics are recommended, with Figure 1.6 illustrating the high frequency of two of these antibiotics which are prescribed to women. Omitting urine culturing may contribute to inappropriate and inaccurate prescription, therapeutic failure, and higher treatment costs, therefore highlighting the importance of

laboratory guided diagnostics over reliance on national epidemiological data alone (Carmona-Cartaya et al., 2022; O'Grady et al., 2018).

Table 1.1: Empirical antibiotics for uncomplicated UTI in adults (adapted from BpacNZ (2021)).

Therapy	Antibiotic	Dosage	Dose frequency	Treatment duration
First-line	Nitrofurantoin – modified release (Macrobid)	100 mg	BID	5 days
	Nitrofurantoin – immediate release (Nifuran)	50 mg	QID	5 days
Alternatives	Cefalexin	500 mg	BID	3 days
	Trimethoprim	300 mg	QID	3 days

QID= once a day, BID= twice daily

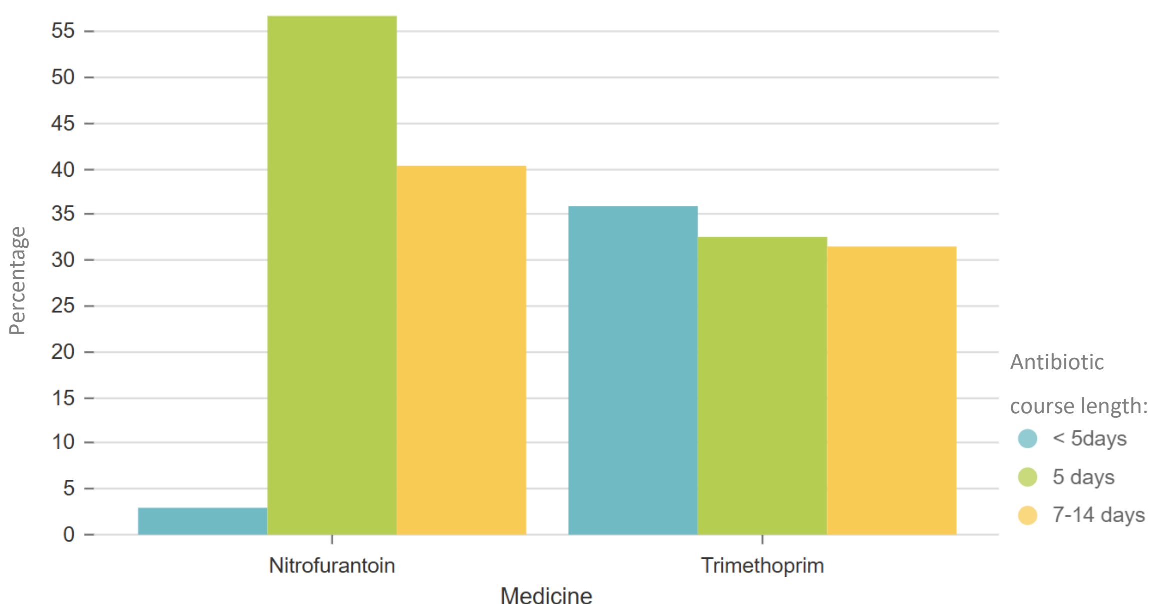

Figure 1.6: Percentage females dispensed nitrofurantoin or TMP for UTI treatment for the year ending December 2024 (retrieved from He Ako Hiringa (n.d)) .

Nitrofurantoin is currently the most prescribed antibiotic for UTI treatment in adults in NZ, whereas other countries such as those in Europe prescribe fosfomycin tromethamine (BpacNZ, 2017; Fajfr et al., 2020). Alternatives such as TMP or cefalexin may be prescribed depending on patient symptoms and infection severity (BpacNZ, 2017). National data shows NZ antibiotic

dispensing rates are higher than most European countries of the same economic status, with nearly every child exposed to antibiotics by the age of five (Duffy et al., 2018; Ministry of Health, 2023; Thomas et al., 2020; Williamson et al., 2016).

If empirical therapy fails (i.e., the patient still has symptoms after the antibiotic course is completed), urine culture an susceptibility are preformed to identify the causative pathogen (Harris & Perencevich, 2007; Le & Miller, 2001; C. H. J. Lee et al., 2018; Novelli & Rosi, 2017). However, urine cultures are omitted if patients respond well to empirical treatment (Nickel, 2005). While empirical guidelines vary internationally, cultures are strongly recommended in recurrent UTIs in cases where patients have conditions such as overactive bladder or vulvodynia, which can mimic UTI symptoms (Bogart et al., 2007; Peng et al., 2021).

1.3.5 Antibiotic Resistance

Antimicrobial resistance (AMR) refers to the ability of microorganisms to survive an exposure to antimicrobial agents, such as antibiotics, at concentrations which are considered lethal to their species (Pattis et al., 2022; Sheikh et al., 2019). The World Health Organisation ranks AMR among the top ten global public health threats, causing over 700,000 deaths annually, and reducing available patient treatment options and outcomes (Ashiru-Oredope & Hopkins; Coll et al., 2024). Rising resistance has made antibiotic treatment challenging as the development of new agents is no longer an efficient or sustainable solution to the rate that bacteria adapt (Uddin et al., 2021). Without intervention, AMR related deaths may reach 10 million by the year 2050, causing a huge economic burden globally (Walsh et al., 2023).

1.3.5.1 Molecular Basis of Antibiotic Resistance

There are two main genetic strategies which allow bacterial adaptation to antibiotics: spontaneous gene mutation and horizontal transfer of resistance genes (Ndagi et al., 2020). Mutations often arise after the exposure to sub-inhibitory antibiotic concentrations which has enabled survival but may disrupt cell homeostasis and reduce bacterial cell fitness (Ndagi et al., 2020; Sultan et al., 2018). In contrast, horizontal gene transfer, via transformation, transduction, or conjugation, is the predominant genetic strategy as it allows bacteria to acquire foreign DNA and enhancing environmental fitness (Lloyd et al., 2009; Munita & Arias, 2016).

At the molecular level, resistance is facilitated by four main mechanisms: enzymatic inactivation or antimicrobials, modification of drug targets, reduced membrane permeability, and active efflux pumps (Figure 1.7) (Gajic et al., 2022; D. S. Lee et al., 2018a). Some bacteria produce enzymes, such as β-lactamases which chemically inactivate antibiotics to make them ineffective (Gajic et al., 2022). Antibiotic target sites may be modified by replacing, protecting, or altering

the sites where they bind (Gajic et al., 2022; D. S. Lee et al., 2018a). Mutations in porin proteins can lower bacterial membrane permeability and limiting antibiotic concentrations entering the cell (Salam et al., 2023). Active efflux pumps expel antibiotics from bacterial cells resulting in sub-lethal antibiotic concentrations within cells and allowing cell survival (Wanda, 2018).

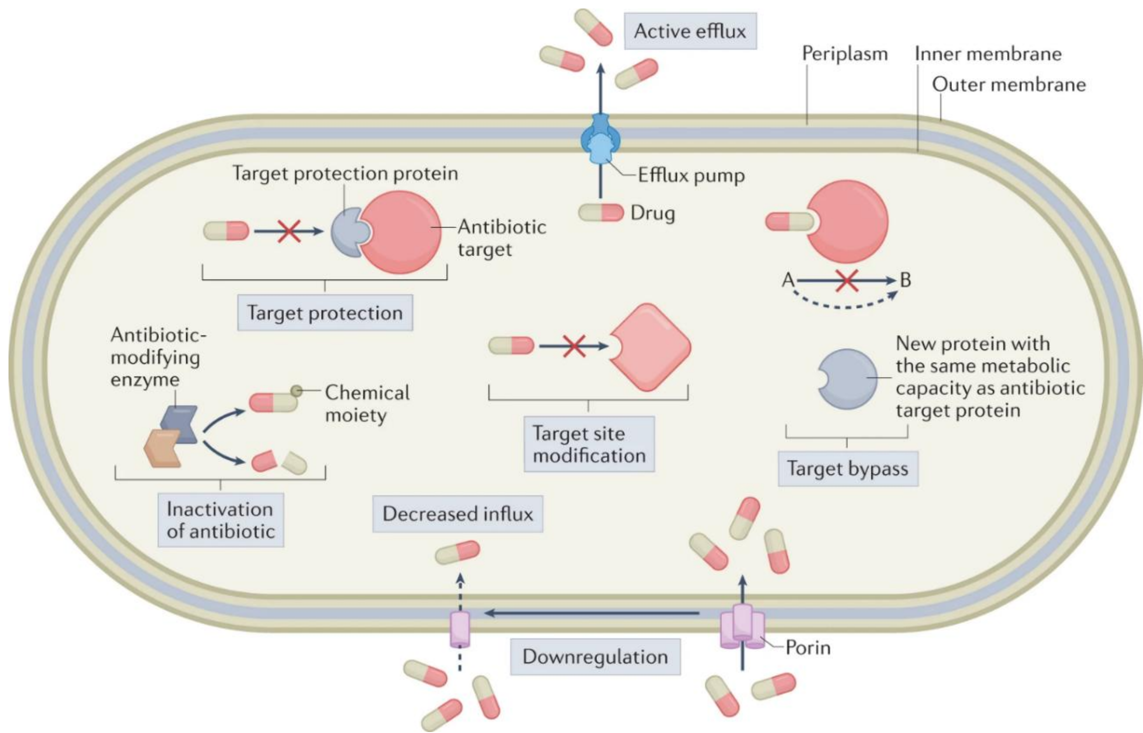

Figure 1.7: General resistance mechanisms against antibiotics in bacterial cells (retrieved from Darby et al. (2022)).

1.3.5.2 Uropathogenic *E. coli* Resistance and Trimethoprim Resistance

While folate metabolism is a powerful target for drug therapies and their development, there has been a large wave of resistance to many antifolates (Kordus & Baughn, 2019). These resistance mechanisms have been identified in clinically important *E. coli* during exposure to high levels if TMP (Somorin et al., 2022).

Antibiotic resistance in UPEC may occur through chromosomal mutations, efflux pumps, enzymatic inactivation, and target site modification (Whelan et al., 2023). Horizontal gene transfer of virulence factors via plasmids enhances adaptability to antibiotic present environments and enabling the spread of resistance across bacterial populations (Brolund et al., 2010; Grape et al., 2007; Somorin et al., 2022). Increasing global resistance rates have been reported to first-line antibiotics such as TMP-SMX (Schito et al., 2009; Zagaglia et al., 2022; Zhanel et al., 2006). TMP resistance in urine samples has been established by studies to be mediated by *dfrA* genes variants (e.g., *dfrA1*, *dfrA5*, *dfrA7*, *dfrA12*, or *dfrA17*) and occasionally

by the less frequent homologue type B DHFRs (*dfrB*) (Brolund et al., 2010; Cellier-Goetghebeur et al., 2022; Grape et al., 2007; Somorin et al., 2022). Another key resistance mechanism involves the acquisition of an additional TMP insensitive DHFR which is encoded by *dfrA* genes on mobile elements, which enables folate reduction under antibiotic stress (Adrian & Klugman, 1997; Myoda et al., 1984).

As presented in Figure 1.8, TMP inhibits the chromosomal encoded FolA enzyme, limiting the THF cycle. Therefore, resistance can also result from increased *folA* expression or amino acid substitutions which reduces TMP binding, therefore maintaining THF synthesis and carbon metabolism (Huovinen et al., 1995; Kordus & Baughn, 2019; Sköld, 2000). Chromosomal mutations which upregulate *folA* transcription may also contribute to TMP resistance (Kordus & Baughn, 2019; Sánchez-Osuna et al., 2020).

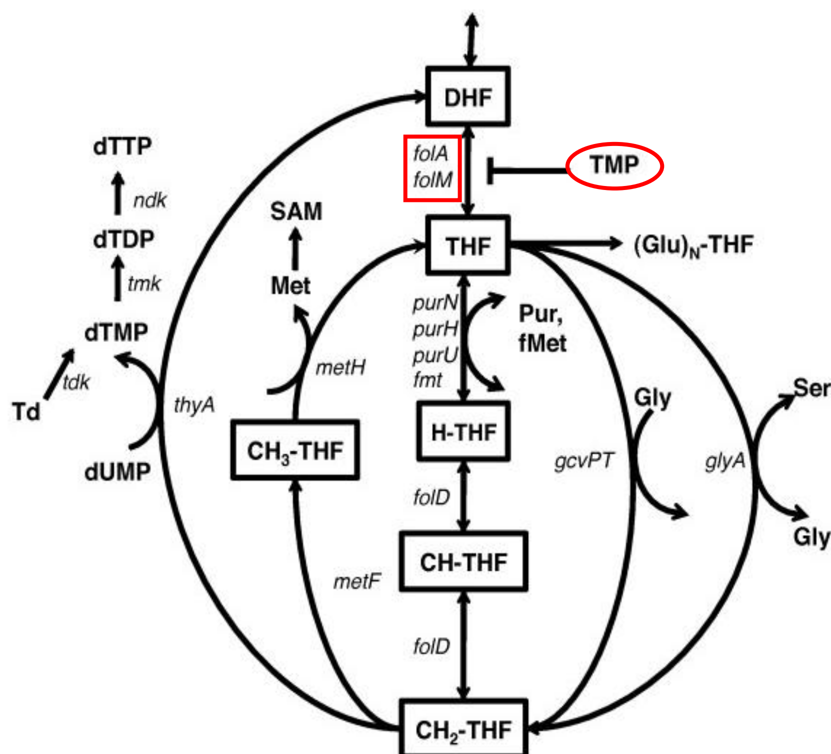

Figure 1.8: Overview of the THF metabolism encoded by the folA and folM genes in E. coli (adapted from Sangurdekar et al. (2011)).

Target site replacement is another known resistance mechanism to TMP-SMX through dihydropteroate synthase (DHPS) overproduction. Normally, DHPS catalyses the formation of dihydrofolate (DHF) from para-aminobenzoic acid (PABA), a process which is inhibited by SMX (as shown in Figure 1.5), whereas resistance can arise through mutations in the DNA coding region of DHFR (Minato et al., 2018). DHPS overproduction results in excess production of enzymes which diminishes TMP-SMX ability to inhibit bacterial folate synthesis, and alows bacterial survival (Eliopoulos & Huovinen, 2001; Flensburg & Sköld, 1987; Ndagi et al., 2020).

Resistance evolution to TMP may occur through the alteration of the binding target through sequential accumulation of resistance-conferring mutations in the DHFR enzyme in bacterial cells (Schober et al., 2019; Tamer et al., 2019). Recent whole genome studies of gram-negative bacteria several new TMP resistant genes encoding a FolA homologue have been found with some showing to experimentally confer TMP resistance (Ambrose & Hall, 2021a).

Antibiotics causing DNA damage such as TMP, have been shown to induce a stress response resulting in the elevation of *recA* expression (Uhlich et al., 2018). Other AMR mechanisms may involve bacterial envelope impermeability therefore, suggesting other housekeeping genes may be involved in TMP resistance for the repair of recombinational DNA during antibiotic stress (El-Halfawy & Valvano, 2012). These identified mechanisms and associated genes have been listed in Table 1.2.

Table 1.2: Mechanisms of intrinsic TMP resistance which have been investigated.

Resistance mechanism	Example	Organism	Reference
Antifolate-target mutations	*dfrA*	*E. coli*, *Klebsiella* spp., *P. mirabilis*	Brolund et al. (2010); Somorin et al. (2022)
	dfrA	*E. coli*	Ambrose and Hall (2021b); Grape et al. (2007); Schmidt et al. (2019); Wüthrich et al. (2019)
	dhps (*folP*) gene	*E. coli*, *Campylobacter*, *Streptococcus pneumoniae*, *Neisseria meningitidis*	Sköld (2000)
Antifolate-target isoforms	DfrB	*E. coli*	Cellier-Goetghebeur et al. (2022)
	sul1 & *sul2*	*Enterobacter* spp.	Alekshun and Levy (2007)
	DHPS	*E. coli*, *Campylobacter*, *Streptococcus pneumoniae*, *Neisseria meningitidis*	Sköld (2000)
Reduction of cell permeability	Increase of efflux pump proteins	Many bacterial species	Kordus and Baughn (2019); Then (1982)
		Pseudomonas aeruginosa	Hamilton-Miller (1979)
		E. coli	Pato and Brown (1963)
Overexpression of target	FolA	*E. coli*	Kordus and Baughn (2019)
	DHFR	*E. coli*	Grey et al. (1979); Sköld (2000)
Degradation	Polyglutamation	Human cells	Zhao and Goldman (2003)
Auxotrophy	Thymidine	*E. coli*	Jakovljev et al. (2022)
	Thymidine	Many bacterial species	Maskell et al. (1978); Then (1982)

1.3.5.3 New Zealand Clinical Resistance Rates

Treatment failures, longer hospital stays, and the use of higher antibiotic doses are the burdens caused by the rising AMR rates in hospital and community settings (van Hecke et al., 2017). In NZ, rising AMR has reduced the effectiveness of the first-line antibiotics for UTI treatment, as previously mentioned in Table 1.1 (BpacNZ, 2017). Antibiotic use per-capita is also higher in NZ than countries of similar economic status, particularly for narrow-spectrum penicillins (BpacNZ, 2017; Hobbs et al., 2017). Between the years 2006 and 2014, the use of TMP and TMP-SMX increased, which may have contributed to rising resistance rates (Ussher et al., 2020). Consequently, TMP is no longer recommended for uncomplicated cystitis treatment in NZ (BpacNZ, 2021).

Surveillance data highlights this concern, where 68% of *E. coli* isolates from Auckland City Hospital were resistant to TMP, with resistance also seen in 36% of extended spectrum beta-lactamase (ESBL) *E. coli* (LabPLUS, 2023) (Table 1.3). Community data show similar trends, with TMP resistance in *E. coli* rising from 19% in the year 2000 to approximately 25% in the year 2023 (Mangin et al., 2005; New Zealand Formulary, 2023). However, AMR prevalence remains lower in NZ than some regions of the globe (Arden et al., 2024).

Table 1.3: Resistance results for *E. coli* isolates from adult and paediatric clinical specimens in 2022 (adapted from LabPLUS (2023)).

≥ 90% susceptible; 70-89% susceptible; < 70% susceptible; Number = proportion susceptible (S/I); R = Resistant

Classificat on Number: LPMICCOMIN008 Date of issue: Feb 2023 Organism (number tested)	Amoxycillin	Amoxycillin-clavulanate	Cephalexin[c]	Cefuroxime	Ceftriaxone	Ceftazidime	Piperacillin-tazobactam	Aztreonam	Meropenem	Gentamicin	Amikacin	Trimethoprim[c]	Cotrimoxazole	Ciprofloxacin	Norfloxacin[c]	Nitrofurantoin[c]
Escherichia coli – all (3757)	41	81	77	83	90	89	92	90	100	89	99	68	70	80	76	99
ESBL *Escherichia coli* (307)[a]	R	-	R	R	R	R	-	R	100	56	99	36	37	24	12	97

Department of Clinical Microbiology, Auckland City Hospital.

1.3.5.4 Global Spread of UPEC Resistance

Antibiotic resistance particularly in UPEC is a major global health concern, contributing to treatment failures, prolonged hospitalisation, and the increase in administered antibiotic doses (van Hecke et al., 2017; Zeng et al., 2021). The global prevalence of resistance strains may be accelerated by globalisation, making AMR a worldwide burden, regardless of geographical boarders (Carmona-Cartaya et al., 2022; Seoane & Bou, 2021). UPEC and other bacteria develop intrinsic resistance and spread determinants through horizontal gene transfer, allowing rapid adaptation under antibiotic pressure (Sultan et al., 2018).

1.3.6 Antibiotic Susceptibility Testing

Antimicrobial susceptibility testing (AST), described in Section 1.3.3.2, uses phenotypic methods to assess the drug susceptibility of pathogens and guide antibiotic treatment (van Belkum et al., 2020). Depending on the infection type, turnaround time, cost and resources, the methods used by laboratories as shown in Figure 1.9.

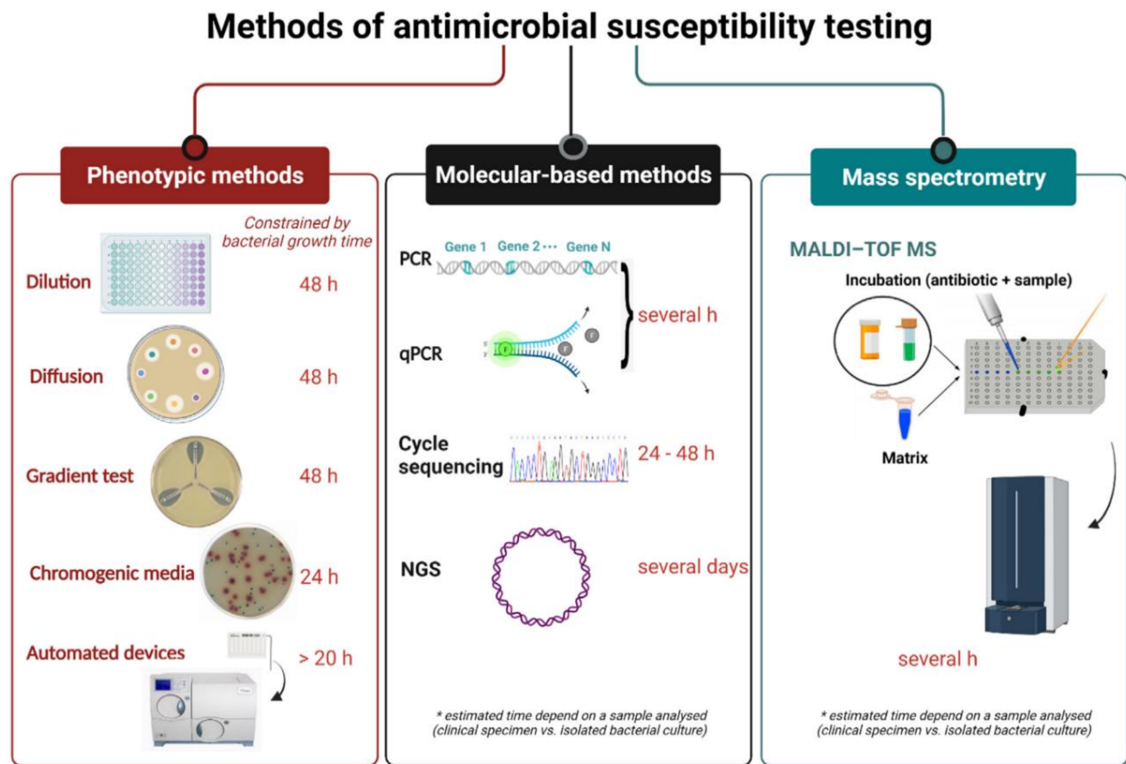

Figure 1.9: Methods used for AST with the turnaround time (retrieved from Gajic et al. (2022)). PCR- polymerase chain reaction, qPCR- quantitative polymerase chain reaction, NGS- next generation sequencing, MALDI-TOF MS – matrix-assisted laser desorption/ionisation time-of-light mass spectrometry.

1.3.6.1 Disk Diffusion Assays

Disk diffusion assays are interpreted by assessing the presence or absence of visible bacterial growth around an antibiotic impregnated disk and is referred to as the zone of inhibition (EUCAST, 2023). Test organisms are classified as susceptible, susceptible at an increased exposure (intermediate), or resistant to an antibiotic according to international guidelines set as breakpoints (CLSI, 2023; EUCAST, 2022). These guidelines allow for standardisation into categorical resistance levels as zones of inhibition diameters differ depending on the species and antimicrobial agent being tested (CLSI, 2023; EUCAST, 2022; Matuschek et al., 2014). Antibiotic disks are commercially available and low cost, compared to molecular testing, and are often reliable and reproducible, however is limited by the time needed for incubation to obtain colony

growth (Bertranda, 2019; Gajic et al., 2022). Disk diffusion AST is widely used in laboratory diagnostics to detect resistance and guide appropriate antibiotic prescription (van Belkum et al., 2020).

1.3.6.2 Broth Dilution Methods

Macro and microbroth dilution are one of the earliest methods of AST which involve preparing two-fold antimicrobial dilutions in liquid media to determine the minimum inhibitory concentration (MIC), which is the lowest concentration which visibly inhibits the test organisms growth after a 24 hour incubation period (Jorgensen & Ferraro, 2009; Levison & Levison, 2009). MICs vary depending on species, strains and the antimicrobial agents being tested (Levison & Levison, 2009; Wald-Dickler et al., 2018). In AST, isolates which are classified as susceptible by disk diffusion assays generally have low MICs, whereas resistant isolates typically have high MICs, with breakpoints distinguishing between susceptible and resistant populations (Figure 1.10) (EUCAST Definitive Document, 1998; Kowalska-Krochmal & Dudek-Wicher, 2021; Levison & Levison, 2009). MIC testing is reproducible and offers a quantitative measurement of resistance beyond what is offered by disk diffusion assays (Diaz-Diaz et al., 2022; EUCAST, 2023; Hattab et al., 2024).

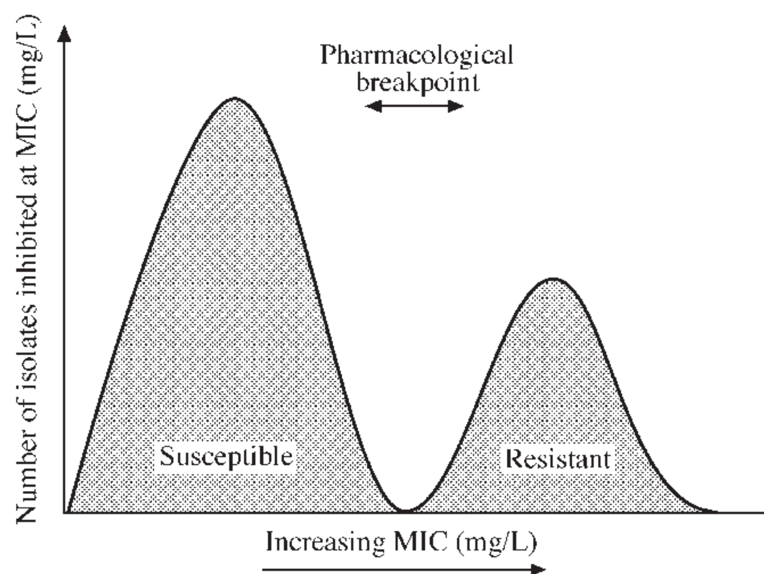

Figure 1.10: Interpretation of antibiotic breakpoint (retrieved from MacGowan and Wise (2001)).

Macrobroth Minimum Inhibitory Concentration

Macrobroth MIC dilution assays are typically performed in test tubes with larger volumes of media, enabling the direct visualisation of bacterial growth, as suggested by turbidity, across tubes of antimicrobial concentrations (Andrews, 2001). This method is often used when validating new breakpoints (Jorgensen & Ferraro, 2009). It is widely used in research and for quality control however, despite its accuracy, microbroth MIC testing is not used in routine clinical diagnostics due to the labour and reagent costs (Balouiri et al., 2016).

Microbroth Minimum Inhibitory Concentration

Microbroth MIC dilutions are performed in small volumes which include broth and bacterial suspensions tested in 96-well microtiter plates (Krishnamurthi et al., 2021). Growth is measured using spectrophotometers or 96-well plate readers to measure optical density (OD) at a wavelength of 600 nm (Krishnamurthi et al., 2021; Mira et al., 2022). The growth curves which are generated from ODs provide a simple and reliable method used to monitor microbial replication and assess bacterial growth under antibiotic conditions (Andrews, 2001; Krishnamurthi et al., 2021). Time-kill studies may also be done by measuring bactericidal activities through changes in CFU/mL over 24 hours, which allows for the calculation of the minimum bactericidal concentration (MBC) and is defined as a $\geq 10^3$ fold reduction in bacterial density (Wald-Dickler et al., 2017). Overall, OD based assays and broth dilutions are generally reliable and reproducible but remain low throughput and are time consuming as overnight incubation is needed (van Belkum et al., 2020).

1.3.7 Whole Genome Sequencing

As mentioned in Section 1.3.5.1 bacteria have complex biochemical pathways to survive antimicrobial agents, many of these involve complex gene networks which can remain poorly understood (Seoane & Bou, 2021; Sultan et al., 2018). Whole genome sequencing (WGS) is a powerful tool used to investigate these pathways by offering rapid, consistent, and accurate predictions of resistance phenotypes across the entire bacterial genome (Tyson et al., 2015). WGS involves culturing an isolating the organism, extracting genomic DNA to sequence and assemble the genome for analysis (Seoane & Bou, 2021; Su et al., 2019). This technology has facilitated the identification and epidemiological tracking of clinical pathogens such as *E. coli*, while identifying AMR genes (Hilt & Ferrieri, 2022). When compared to phenotypic methods such as AST, WGS has a higher resolution and greater capacity for cataloguing AMR associated genes and potential resistance mechanisms in an organism (Seoane & Bou, 2021; van Belkum et al., 2020).

1.3.7.1 Next Generation Sequencing

Next generation sequencing (NGS) enables rapid identification of resistance genes within genomes and metagenomes (Boolchandani et al., 2019). Commonly used technology includes Illumina (San Diego, USA) and Oxford Nanopore Technologies (Oxford, UK).

Oxford Nanopore Technologies and Nanopore MinION

The Nanopore MinION from Oxford Nanopore Technologies (ONT, Oxford, UK) involves passing single strands of DNA or RNA through nanopores which are embedded in an electrically resistant membrane (Hilt & Ferrieri, 2022). Nucleotide specific disruptions in current are translated into base sequences using computational algorithms or basecalling (Figure 1.11) (Giordano et al., 2017; Hu et al., 2021; Su et al., 2019). ONT offers high throughput, rapid turnaround, and produces long reads of ≥ 10 kb which enables fewer gaps in reads, better resolution of repeat regions, and the detection of plasmids and large genomic rearrangements (Giordano et al., 2017; Hu et al., 2021; Su et al., 2019; van Belkum et al., 2020). However, this is limited by the need for high molecular weight DNA or RNA which are of a high quality to maximise the accuracy of sequencing reads (Jones et al., 2021).

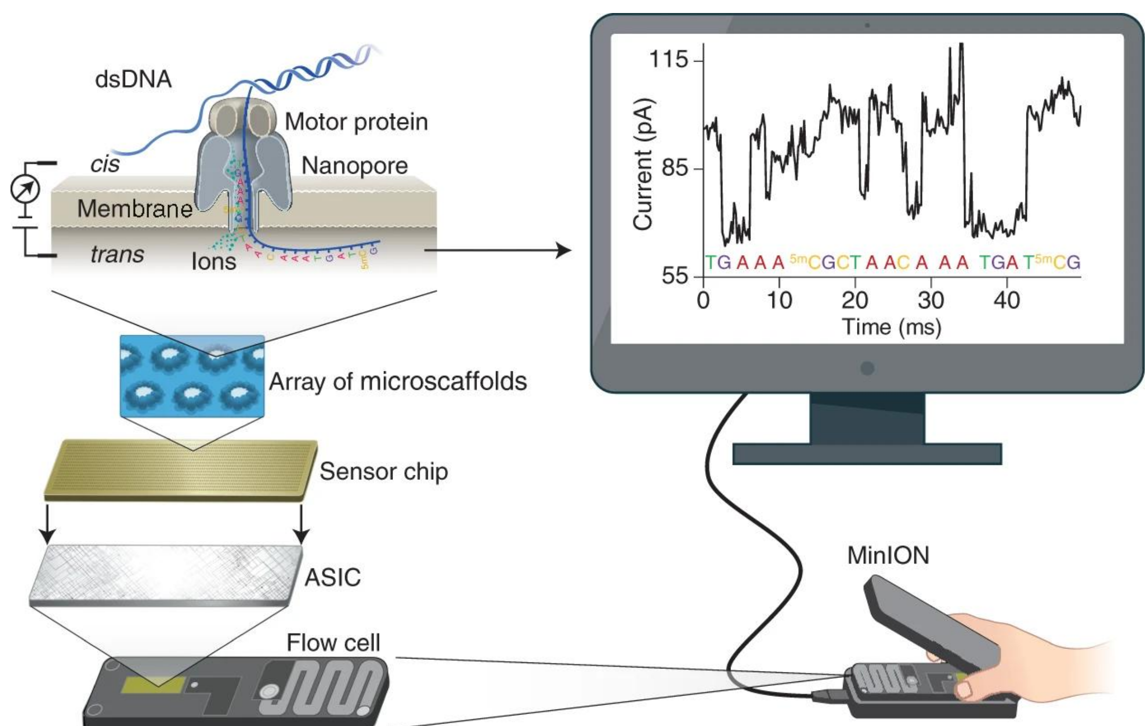

Figure 1.11: ONT MinION sequencing principle (retrieved from Wang et al. (2021)).

1.3.8 Bioinformatics and Comparative Genomics

Bioinformatics applies computational methods such as sequence and structural alignments to analyse biological data and contributing to data and knowledgebases (Luscombe et al., 2001). Expanding molecular and literature databases advances pathogen epidemiology and AMR research (Carriço et al., 2013). These tools support the investigation of resistance mechanisms, enabling the identification and classification of causative pathogens, and detect diagnostic and therapeutic determinants to direct patient treatment (Saeb et al., 2017).

Comparative genomics has been utilised to investigate genetic differences between antibiotic sensitive and resistance phenotypes, such as sequencing UPEC to study virulence and AMR patterns (Hossain et al., 2020; Qin et al., 2018; Sung et al., 2024). While UPEC pathogenesis is well studied, the roles of many *E. coli* genes during uncomplicated UTI treatment remain unclear (Subashchandrabose et al., 2014). Utilising comparative genomics can help understand if specific genomic features have clinical outcomes, therefore, providing insight into resistance evolution (Kao et al., 2018; Thänert et al., 2019).

1.4 Summary of Literature Review

Overall, this chapter has discussed the predominant uropathogen, UPEC and the global concern associated with its association to AMR. The rise in global rates of TMP resistance has demoted its use as a first line antibiotic treatment for UTIs. Research contributing to UPEC resistance from NZ isolates is scarce. To address these gaps, this study will combine AST to determine which antibiotics UPEC isolates are resistant to (Research Question 1), with growth curves to assess how TMP resistance influences growth rates under differing concentrations (Research Question 2). Finally, WGS and comparative genomics will be used to identify genetic differences among isolates of differing levels of TMP (Research Question 3) to provide insights into AMR mechanisms of UPEC.

Chapter 2 Antibiotic Susceptibility Testing of *E. coli*

2.1 Introduction

Urinary tract infections (UTIs) are among the most common bacterial infections globally, affecting both communities and healthcare settings and frequently requiring antibiotic treatment (Wagenlehner et al., 2020; Zagaglia et al., 2022; Zhou et al., 2023). However, rising resistance has reduced the effectiveness of many antibiotics and influenced management strategies (Schito et al., 2009). Trimethoprim (TMP), once a widely used for UTI management, is considered a redundant antibiotic due to high levels of global resistance (D. S. Lee et al., 2018a).

As outlined in Section 1.3.5.4 antibiotic susceptibility testing (AST) remains the gold standard for determining organism susceptibilities to antimicrobial agents (EUCAST, 2023). Disk diffusion assays are widely used in clinical and experimental settings due to their simplicity and reproducibility; however, they are limited by their inability to quantify the concentrations which inhibit growth (EUCAST, 2021; Jorgensen & Ferraro, 2009).

Laboratory screening and diagnosis is determined by AST and accounting for antimicrobial resistance (AMR) phenotypes, which in return guides patient treatment (Wenzler et al., 2023). New Zealand (NZ) based studies have shown the burden caused by *E. coli* AMR within hospitals and in the environment (Cookson et al., 2022; Heffernan et al., 2009; Pattis et al., 2022). Data from Auckland Hospital's annual AMR surveillance, along with the most recent national study, highlight *E. coli* as a significant concern for antibiotic management, particularly due to the prevalence of extended-spectrum β-lactamase (ESBL) producing *E. col*i, which reduces antibiotic options Table 2.1. This concern is further aggravated by NZ demonstrating high antibiotic dispensing rates per capita, which exceeds most European countries (Williamson et al., 2016).

Table 2.1: Percentage of *E. coli* isolates susceptible to antibiotics.

Study	LabPLUS (2023)		Heffernan et al. (2009)
	E. coli (3757)	**ESBL *E. coli*** (307)	**ESBL *E. coli*** (55)
Meropenem	100	100	100
Trimethoprim	68	36	27
Amoxicillin-Clavulanic Acid	81	-	47
Nitrofurantoin	99	97	95

Red is < 70% susceptible, orange is 70-89% susceptible, green is ≥ 90% susceptible. ESBL, extended spectrum β-lactamase.

Minimum inhibitory concentration (MIC) testing is done to provide a quantitative measure of resistance to antimicrobials (Hossain, 2024). In research, disk diffusion assays are often done for initial screening or confirmation of resistance, followed by MIC testing to determine the concentration which inhibits organism growth (Karami et al., 2015). Phenotypic observations from optical density (OD) growth curves, combined with statistical analysis further allows for quantitative interpretation of MIC results by using specific cut offs of inhibition (Axelsson et al., 2024). Resistance and susceptibility can be interpreted when comparing organism growth in the presence and absence of an antibiotic, as presented in Figure 2.1, where isolates grew at concentrations exceeding the clinical breakpoint of ≥ 32 µg/mL, and demonstrated high levels of resistance (CLSI, 2023; Turnidge et al., 2006; Vinchhi et al., 2023).

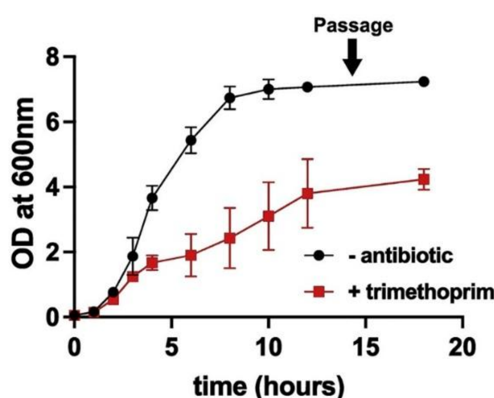

Figure 2.1: Growth characteristics of an *E. coli* strain in the presence (red) and absence (black) of TMP (retrieved from Vinchhi et al. (2023)).

Current research in NZ has not conducted MIC studies following disk diffusion assay AST; instead, studies have primarily reported the resistant isolates observed. This chapter therefore aims to investigate the growth characteristics of TMP resistant *E. coli*, identified through disk diffusion assays and MIC testing in the presence of high concentrations of TMP past the clinical breakpoint of ≥ 32 µg/mL. This study is outlined in Figure 2.2: Schematic of the RQs of this study. Chapter 2 RQs and aims are highlighted in red. Figure 2.2 and further described in Figure 2.3.

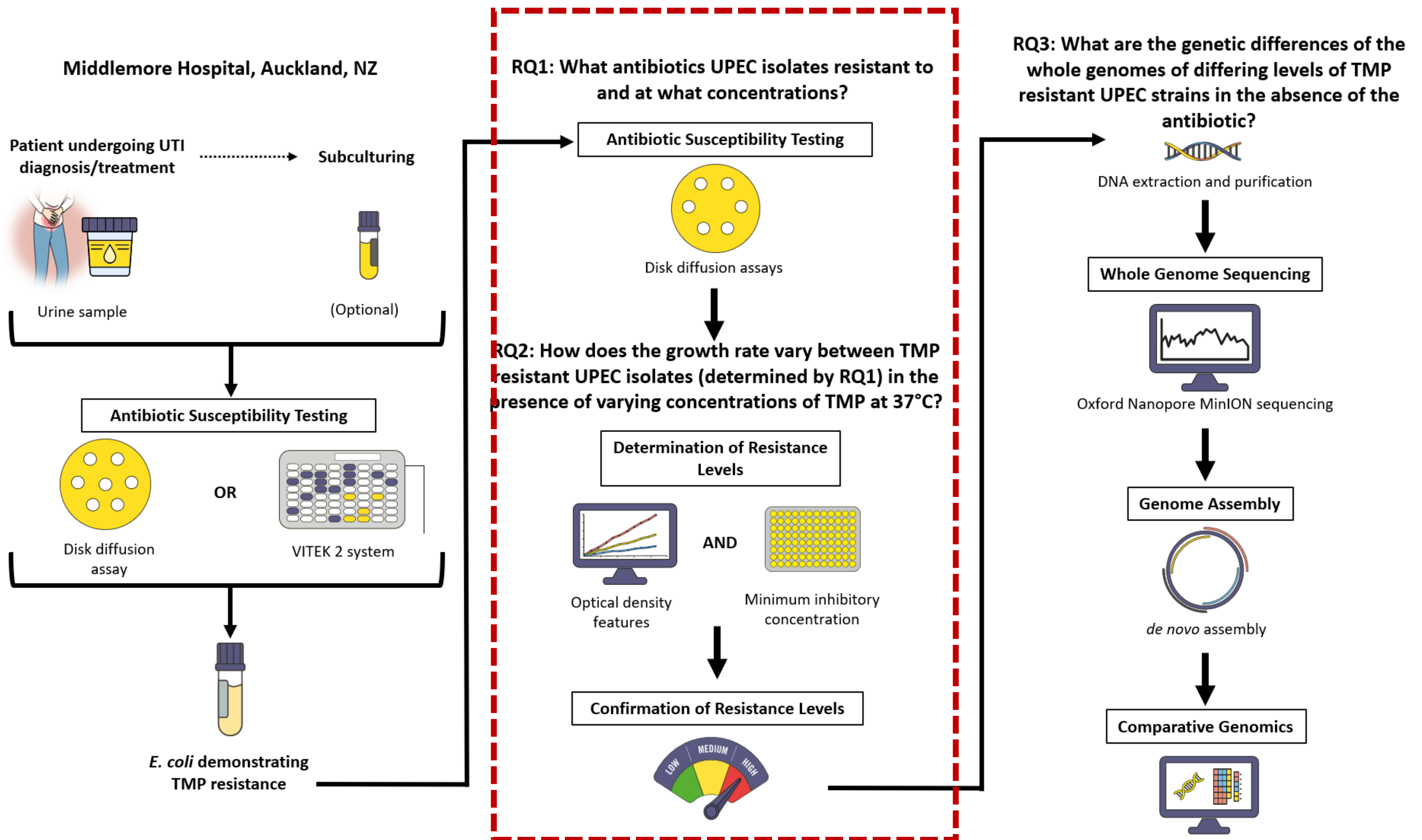

Figure 2.2: Schematic of the RQs of this study. Chapter 2 RQs and aims are highlighted in red.

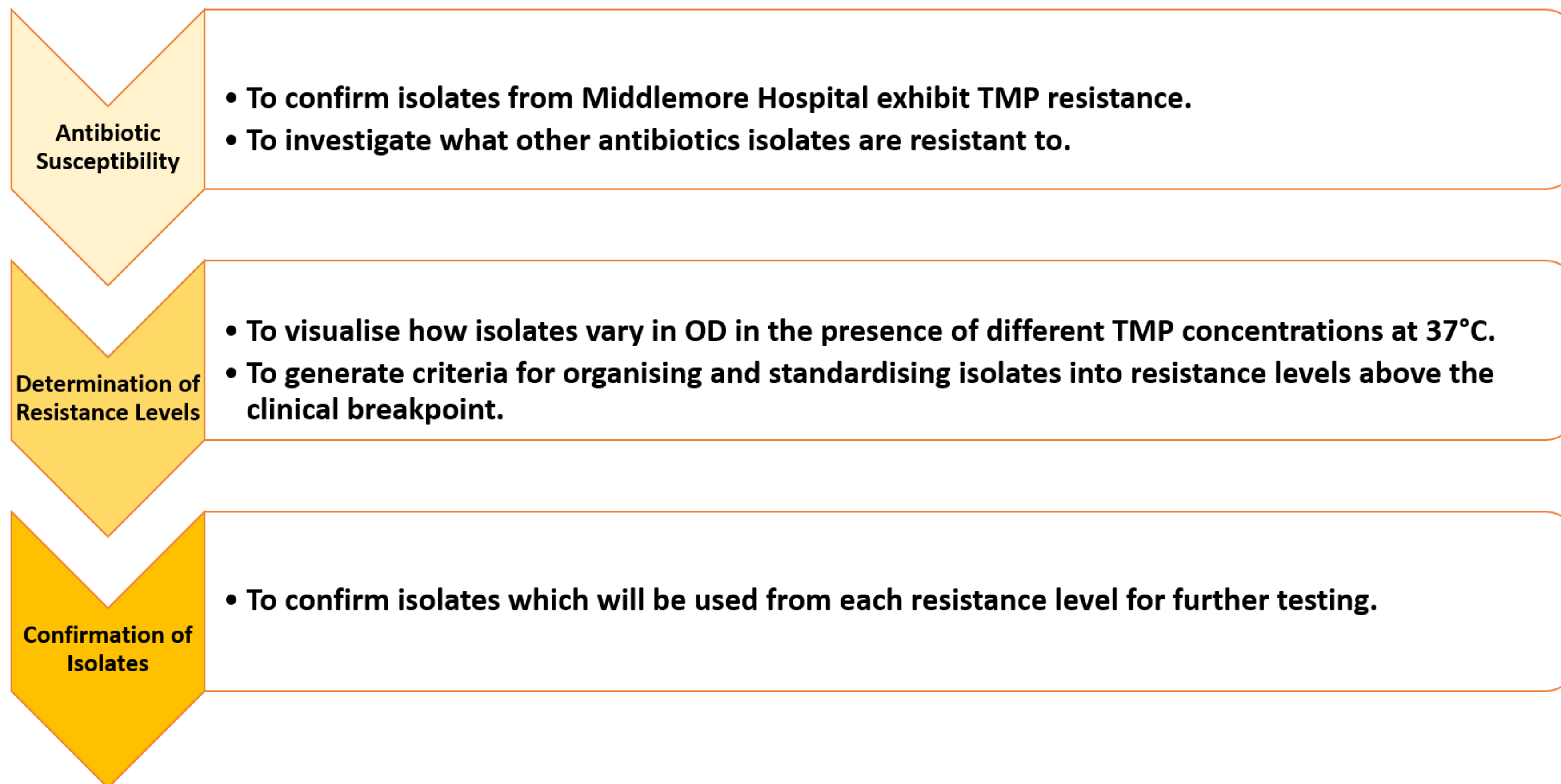

Figure 2.3: Chapter 2 method objectives

2.2 Methods and Materials

Escherichia coli ATCC 25922 was used as a susceptible control strain for testing done at Auckland University of Technology (AUT) as recommended by international guidelines (CLSI, 2023; EUCAST, 2022). Overnight cultures were prepared for each isolate by inoculating 10 mL of antibiotic free Luria-Bertani (LB) broth (prepared according to manufacturer's protocol and pH adjusted) with 100 µL of the urine sample from Middlemore Hospital and incubated at 37 °C for 18-20 hours (CLSI, 2023). Overnight cultures were stored at -80 °C in 20% glycerol (final weight/volume) in a 1.5 mL cryovials until later use.

2.2.1 Initial Testing and Acquisition of Trimethoprim Resistant Isolates

Patient urine samples were provided to Dr Susan Taylor at Middlemore Hospital (Auckland, NZ) and sub-cultured as required depending on sample turbidity.

Antibiotic susceptibility was determined at the hospital laboratory using disk diffusion assays or the VITEK 2 system (bioMérieux), with full panel of antibiotics presented in Appendix A: Middlemore Hospital Antibiotic Susceptibility Testing. TMP resistance was defined by a zone of inhibition of ≤10 mm using a 5 µg TMP antibiotic disk or as resistant by the VITEK 2 system.

Bacterial isolates were identified as *E. coli* based on chromogenic agar pigmentation, indole and oxidase tests results, or via a biochemical card. *E. coli* confirmation was made by matrix-assisted laser desorption/ionisation time-of-light (MALDI-TOF).

Urine samples which demonstrated TMP resistance and where *E. coli* was identified as the causative pathogen, were then aliquoted into 1.5 mL cryovials and frozen at -20 °C for transfer to AUT. Over a three-month period in 2022, 106 TMP resistant *E. coli* isolates were collected and provided to AUT, where they were stored at -80 °C until further testing.

2.2.2 Confirmation of Antibiotic Susceptibility

Disk diffusion assay AST were performed to confirm TMP resistance in isolates obtained from Middlemore Hospital. Overnight cultures were prepared as stated in Section 2.2. Assays were prepared by spreading 100 µL of each overnight culture was spread onto BD Muller Hinton II agar (MHA; made as per manufacturer's protocol and pH adjusted). Commercial antibiotic disks (Fort Richard, NZ) were arranged as shown in Figure 2.4 and tested in duplicate. Antibiotic disks selected for disk diffusion assays reflected UTI treatment guidelines in NZ (mentioned in Table 1.1) and the *E. coli* resistance rates reported in Table 2.1 . Both TMP (5 µg) and TMP-SMX (1.25-

23.75 µg) disks were chosen due to their high resistance rates and as TMP is the centre of this study (LabPLUS, 2023). Nitrofurantoin (300 µg) was used as it is a first line antibiotic used in NZ for UTI treatment and a small percentage resistance was observed (BpacNZ, 2017; LabPLUS, 2023). Amoxicillin-clavulanic acid (20-10 µg) was selected as both intermediate susceptibility and resistance was reported in *E. coli* (Heffernan et al., 2009; LabPLUS, 2023). Meropenem (10 µg) was chosen as a negative growth control given the high susceptibility by *E. coli* (LabPLUS, 2023). A filter paper disk saturated in sterile water to act as a growth control. Agar plates were then incubated at 37 °C for 18-20 hours and antibiotic susceptibility results were recorded, and zones of inhibition were interpreted based on Table 2.2.

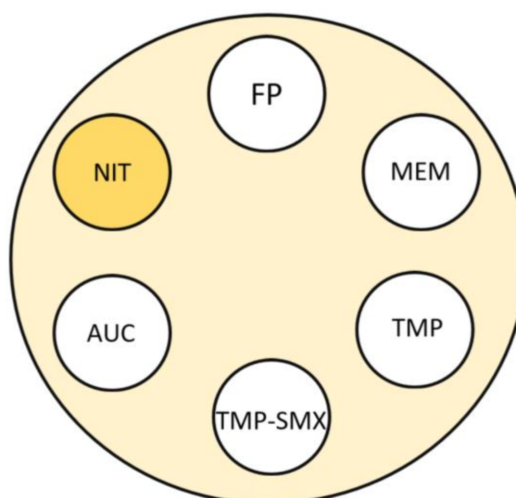

Figure 2.4: Schematic of disk layout on MHA during antibiotic disk diffusion AUT AST.

Table 2.2: Zone diameter and interpretive categories for Enterobacterales as set in CLSI (2023).

		Interpretative Categories and Zone Diameter Breakpoints (nearest whole mm)		
Antimicrobial Agent	**Disk Content**	**Susceptible**	**Intermediate**	**Resistant**
Meropenem	10 µg	≥ 23	20-22	≤ 19
Trimethoprim	5 µg	≥ 16	11-15	≤ 10
Trimethoprim-Sulfamethoxazole	1.25-23.75 µg	≥ 16	11-15	≤ 10
Amoxicillin-Clavulanic Acid	20-10 µg	≥ 18	14-17	≤ 13
Nitrofurantoin	300 µg	≥ 17	15-16	≤ 14

2.2.3 Microbroth Minimum Inhibitory Concentration

As antibiotic disks contain a fixed concentration and cannot quantify resistance levels, microbroth MIC assays were used to assess resistance to increasing TMP concentrations above the clinical breakpoint of 32 µg/mL as stated by CLSI (2023), while providing OD growth rate profiles.

Stock TMP was prepared by dissolving 8192 µg anhydrous TMP (Fort Richard, NZ) in 1 mL sterile Milli-Q water. Overnight cultures were prepared for each isolate by inoculating 10 mL of antibiotic free LB broth (prepared according to manufacturer's protocol and pH adjusted) with 100 µL of the urine sample from Middlemore Hospital and incubated at 37 °C for 18-20 hours (CLSI, 2023). *Escherichia coli* ATCC 25922 was used as a susceptible control strain for testing done AUT as recommended by international guidelines (CLSI, 2023; EUCAST, 2022).

2.2.3.1 Determination of Resistance Levels

From the 83 isolates from Section 2.2.2, which were resistant to TMP and TMP-SMX disk diffusion assays, 74 isolates showed consistent growth up to the antibiotic disks and were selected for further testing using MIC analysis.

Wells in rows B – G (Figure 2.7) in sterile 96-well plates were prepared with 100 µL of sterile Muller-Hinton broth (MHB). Two-fold serial dilutions were carried out from wells one to ten using 100 µL stock TMP (prepared as per Section 2.2.3) to obtain a TMP concentration range of 4096 µg/mL to 8 µg/mL. Respective wells were then inoculated with 5 µL of the overnight culture (prepared as per Section 2.2.3) for a total working volume of 105 µL. Two control wells were present per row: a growth control well consisting of 100 µL MHB and 5 µL of the overnight culture and a sterility control well consisting of 105 µL of MHB.

FLUOstar Omega Plate Reader (BMG LABTECH, Offenburg, Germany) was used to record raw OD values at a wavelength of 600 nm (OD_{600}) every 15 minutes over 24 hours. Growth curves were generated from OD_{600} values using R v4.4.0 via R-studio (R Core Team, 2021). The growth curves were used to identify and generate two criteria: (i) growth rate features and (ii) estimated visual MIC (adapted from (Wiegand et al., 2008)). The 74 isolates were categorised into the TMP resistance levels: low, medium, and high, based on these criteria.

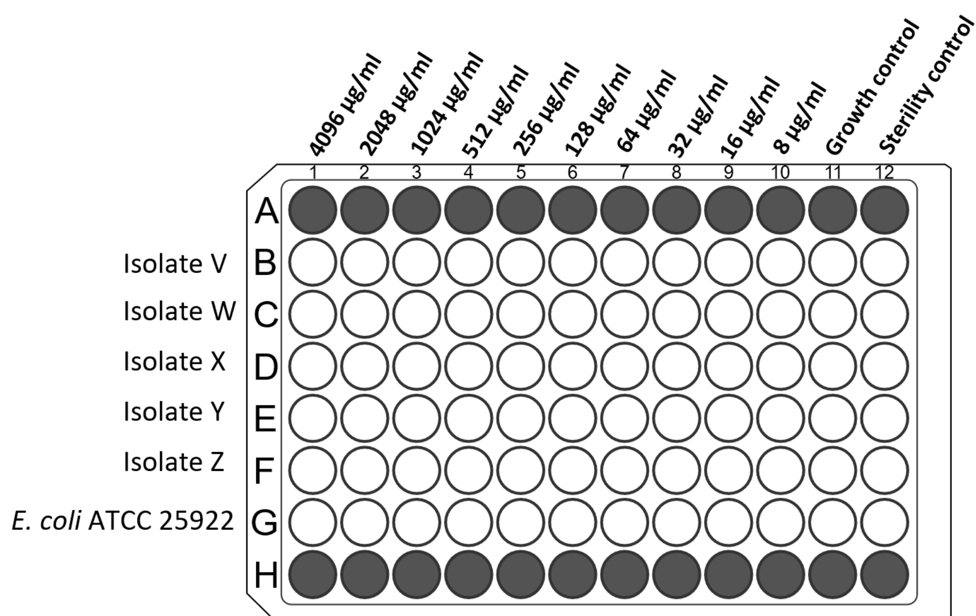

Figure 2.5: Schematic of 96-well plate layout during first round of microbroth MIC testing.

2.2.3.2 Confirmation of Isolates

The growth curves from Section 2.2.3.1 were used to classify isolates into three TMP resistance levels based on the two criteria: (i) growth rate features and (ii) estimated visual MIC. Low resistance was defined by (i) OD_{600} values ≤ 0.750 across all concentrations and (ii) an estimated MIC of ≤ 1024 µg/mL. Medium resistance was defined by (i) OD_{600} values ≥ 0.750 at 8-1024 µg/mL and reduced growth at 2048-4096 µg/mL and (ii) an estimated MIC of 2048 µg/mL. High resistance was defined by (i) OD_{600} values > 0.750 at 1024 µg/mL and (ii) an estimated MIC of 4096 µg/mL.

In order to validate the criteria determined by Section 2.2.3.1 and confirm reproducibility of MIC growth curves, four isolates from each TMP resistance level, for a total of 12 isolates, were selected for a second round of MIC testing using the plate layout shown in Figure 2.6. OD_{600} readings were recorded every 15 minutes over 24 hours using FLUOstar Omega Plate Reader. Triplicate averages, total growth area under the curve (AUC), and sterility controls were analysed and visualised by R v4.4.0 via R-studio.

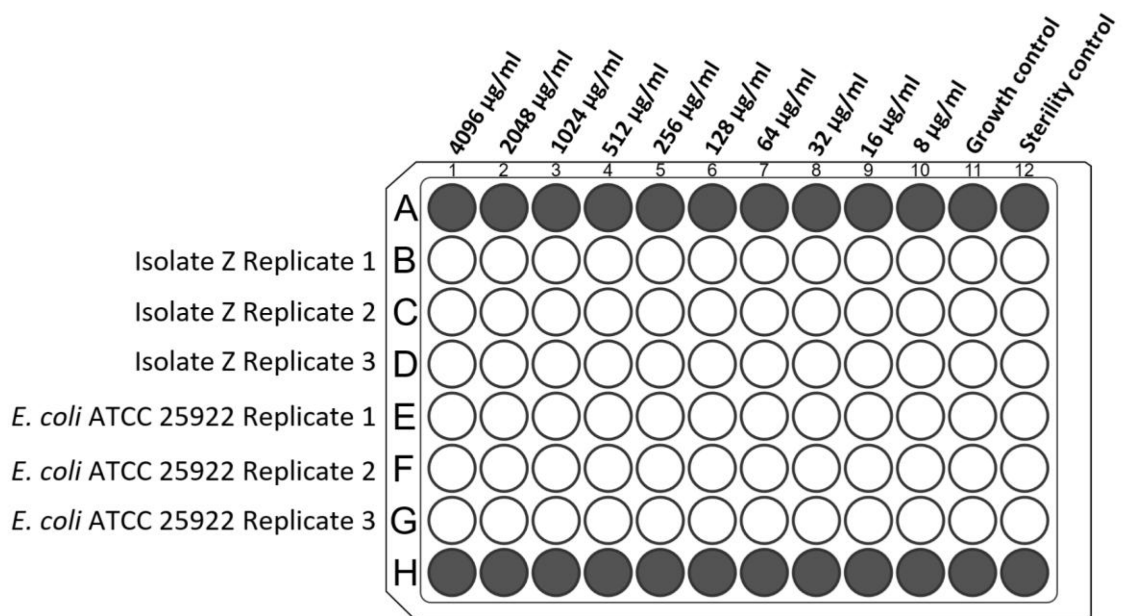

Figure 2.6: Schematic of 96-well plate layout during second round of MIC testing.

2.2.4 Ethics

Ethics approval was not required for the present research. *E. coli* was aliquoted from patient urine by Middlemore Counties Manukau lab before being obtained by AUT. These isolates did not contain urine, human tissue and/or cells identifiable to the patient of origin. Since the present research does not contain any patient identifiable information, or human tissue cells a review by either the Auckland University of Technology Ethics Committee (AUTEC) review and Health and Disability Ethics Committee (HDEC) board in NZ was not required.

2.3 Results

2.3.1 Confirmation of Antibiotic Susceptibility

To confirm the isolates obtained from the Middlemore Hospital demonstrated TMP resistance, disk diffusion assays were performed. Of the 106 isolates, three did not show growth during overnight culturing and were excluded from further testing. The susceptible control strain (mentioned in Section 2.2) showed inhibition to all antibiotic disks and is not discussed further. AST results, according to the breakpoints in Table 2.2, are summarised in Table 2.3.

Among the folate pathway antagonists which were tested, TMP produced the highest number of resistance isolates, with only five of the 103 viable isolates being susceptible. Of the 98 TMP resistant isolates, 83 were also resistant to the combination TMP-SMX, where 74 isolates showed a lawn of growth up to the TMP and TMP-SMX antibiotic disks. Resistance to other drug classes was less common, where one isolate was resistant to meropenem, two to nitrofurantoin,

and eight to amoxicillin-clavulanic acid. Measurements of zones of inhibition may be found in Appendix B: Confirmation of Antibiotic Susceptibility.

Table 2.3: AST sensitivity results of isolates obtained from Middlemore Hospital.

		Sensitivity		
Drug Class	**Antibiotic Disk (concentration)**	**Susceptible**	**Intermediate**	**Resistant**
Carbapenems	Meropenem (10 µg)	101	1	1
Folate Pathway Antagonists	Trimethoprim (5 µg)	5	0	98
	Trimethoprim-Sulfamethoxazole (1.25-23.75 µg)	16	4	83
β-Lactam Combination Agents	Amoxicillin-Clavulanic Acid (20-10 µg)	79	16	8
Nitrofurans	Nitrofurantoin (300 µg)	96	5	2

To determine multi drug resistance (MDR), the resistance profiles of the 103 isolates were analysed across antibiotic classes and respective clinical breakpoints as determined by CLSI (2023). As shown in Table 2.4, most isolates were resistant to one drug class, while 11 isolates exhibited resistance to two antibiotic classes. No isolates were resistant to more than two classes, and five isolates remained fully susceptible to all antibiotic classes tested.

Table 2.4: AST resistance results of isolates to drug classes.

Number of Drug Classes	Number of Isolates
0	5
1	87
2	11
3	0
4	0

2.3.2 Microbroth Minimum Inhibitory Concentration

2.3.2.1 Determination of Resistance Levels

The susceptible control strain (mentioned in Section 2.2.32.2) showed inhibition to all antibiotic disks and is not discussed further. The 74 isolates which demonstrated a lawn of growth up to TMP and TMP-SMX antibiotic disks were analysed to establish criteria for TMP resistance levels above the clinical breakpoint of 32 µg/mL based on generated growth curves. Curves generated for the 74 isolates over the 24-hour period may be found in Appendix C: Determination of Resistance Levels. The two approaches for criteria generation were: (i) growth rate features (Table 2.5) and (ii) estimated visual MIC (Table 2.6). Both methods categorised a higher number of isolates as being in the medium resistance category, with more isolates categorised by the OD growth rate feature criteria. In contrast, only a few isolates were classified as highly resistant for both criteria. All 74 isolates demonstrated an estimated MIC greater than the clinical breakpoint of 32 µg/mL (Table 2.6).

Table 2.5: Isolate criterium for three resistance levels based on OD growth rate features.

Resistance Grading	OD Growth Rate Features	Number of Isolates
Low	- OD ≤ 1.000 for all concentrations	9
Medium	- OD ≥1.000 for 8 -1024 µg/mL - OD ≤1.000 for 2048 µg/mL and 4096 µg/mL	58
High	- OD ≥0.750 for 4096 µg/mL - OD ≥1.000 for other concentrations.	7

Table 2.6: Isolate criterium for three resistance levels based on estimated MIC from growth curves.

Resistance Grading	Estimated MIC	Number of Isolates
Low	≤ 1024 µg/mL	17
Medium	2048 µg/mL	47
High	4096 µg/mL	10

2.3.2.2 Confirmation of Isolates

The susceptible control strain (as mentioned in Section 2.2.3) showed inhibition to all TMP concentrations and is not discussed further. Twelve isolates which matched the criteria for both Table 2.5 and Table 2.6 were used to interpret their respective resistance level. Isolates in the low resistance level showed lower growth curves at the highest TMP concentration (4096 µg/mL) compared to the other two resistance levels. Medium resistance isolates showed high growth curves at 8-1024 µg/mL TMP concentrations and lowered growth curves at 2048-4096 µg/mL concentrations. In contrast, high resistance isolates showed high growth curves at the highest TMP concentration of 4096 µg/mL. Isolate 21 did not demonstrate reproducible growth curves and was therefore excluded from further experiments.

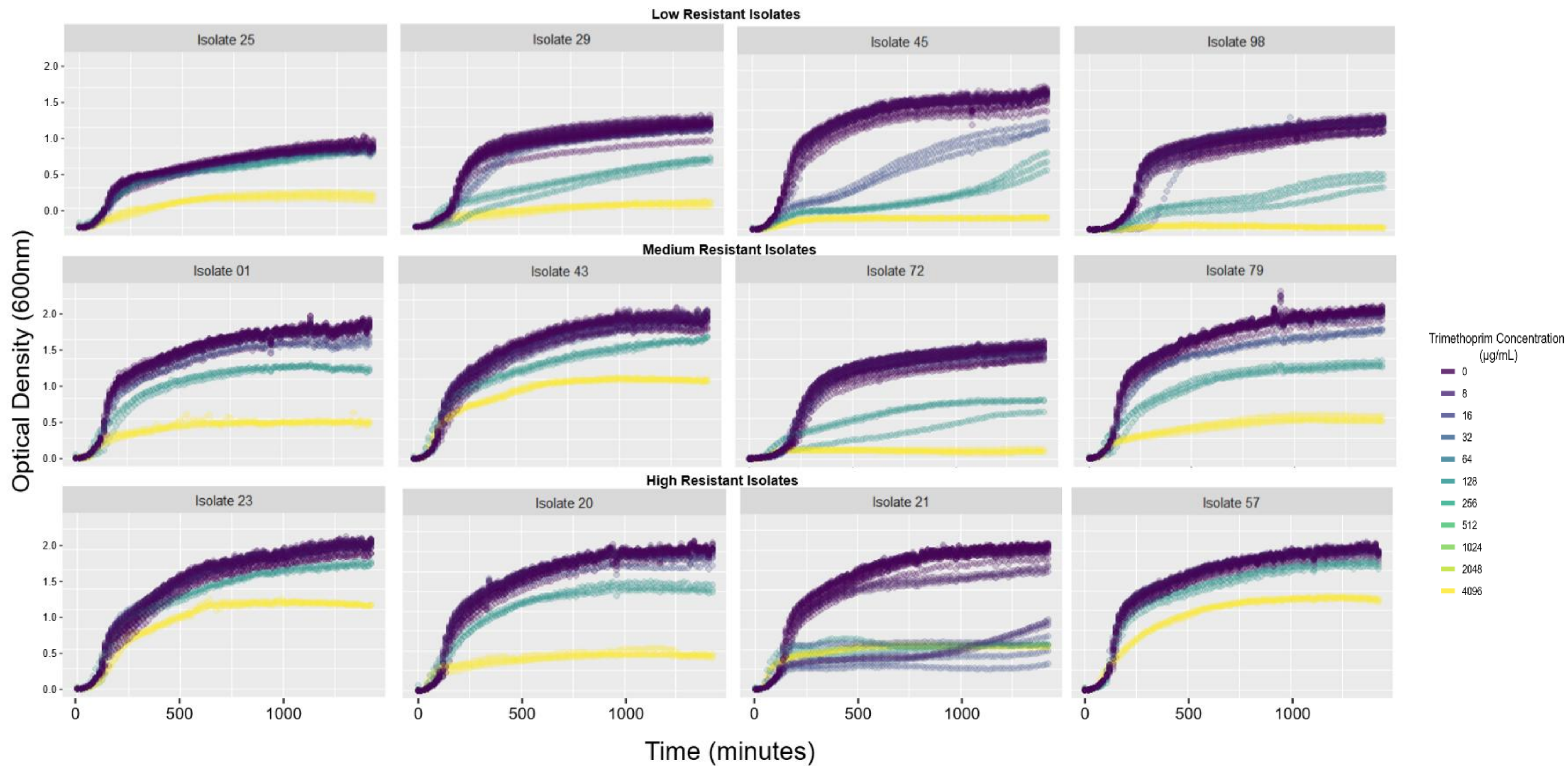

Figure 2.7: OD for triplicate 24-hour readings for the 12 isolates.

Growth of the 12 isolates in the presence of TMP over 24 hours was quantified by calculating the average AUC from replicate OD_{600} values. Isolates are shown in their respective level of TMP resistance; blue (low), yellow (medium), and red (high), with error bars showing the standard deviation across the three biological replicates.

All 12 isolates showed high AUC at TMP concentrations near the clinical breakpoint (grey line). As the TMP concentration increased above the clinical breakpoint, isolates in the low resistance group showed lower AUC than the other two resistance levels. Medium resistance isolates displayed a gradual decline in AUC compared to the high resistance isolates as the TMP concentration increased. In contrast to this, isolate 21 did not follow the same trend at the three other isolates within the high resistance group.

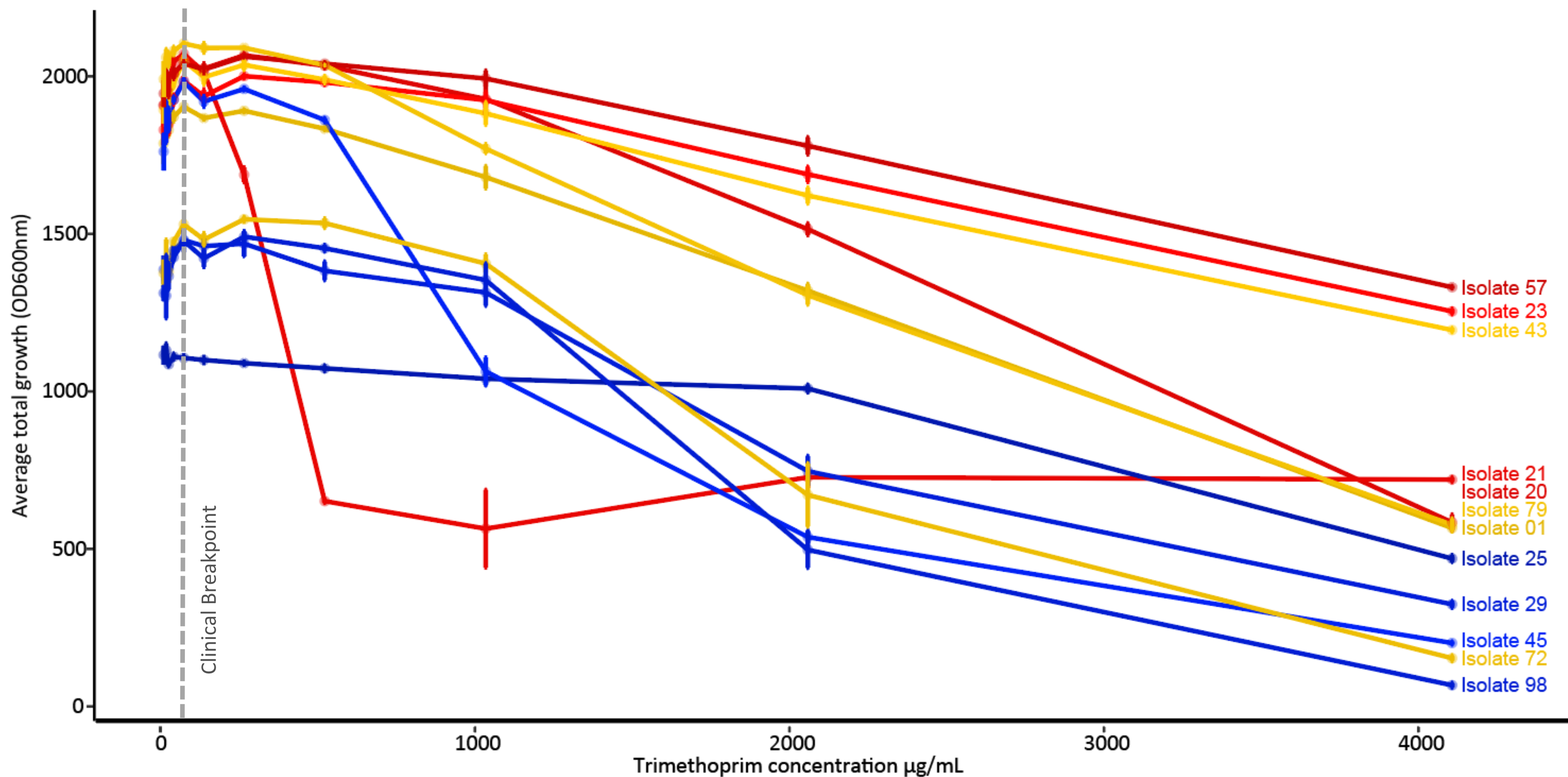

Figure 2.8: Average total growth under curve for the 12 isolates. Blue indicates low TMP resistance, yellow indicates medium TMP resistance, and red indicates high TMP resistance. Clinical breakpoint of 32 µg/mL as stated by CLSI (2023) is shown by grey line.

Figure 2.9 shows the normalised OD_{600} over time for sterility control wells for each of the 12 isolates. Most isolates exhibited values close to zero during the incubation period, however, isolates 20 and 29 showed fluctuations above zero.

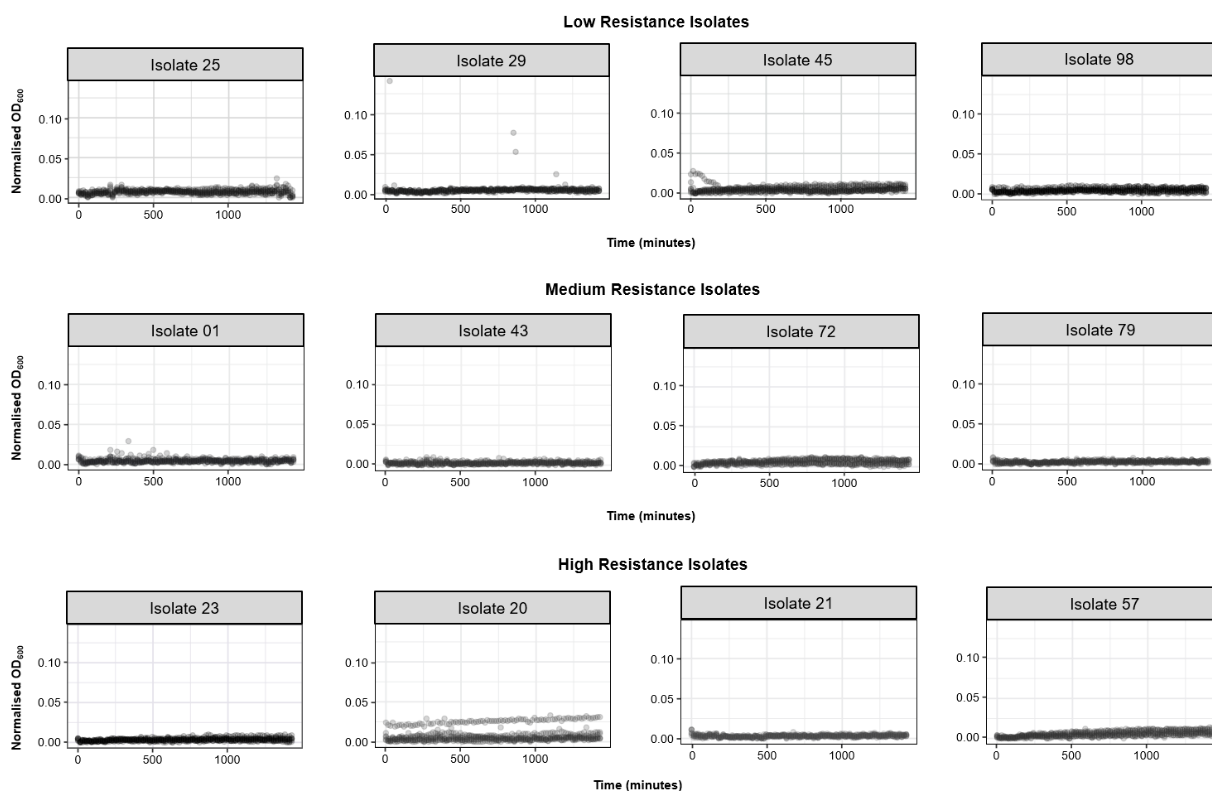

Figure 2.9: Normalised OD for sterility wells for the 12 isolates.

2.4 Discussion

2.4.1 Confirmation of Antibiotic Susceptibility

Patients with UTIs are commonly treated with antibiotics, therefore the information obtained from disk diffusion assay AST plays an important role in guiding patient antibiotic management (Pierce et al., 2023; Zhou et al., 2023).

Disk diffusion assay AST confirmed TMP resistance in 98 isolates, with 83 isolates further exhibiting resistance to the combination TMP-SMX. Resistance to TMP and its combination (TMP-SMX) has been commonly seen in studies as they exert the same selective pressure by targeting different enzymes within the folate biosynthesis pathway (Grape et al., 2007). However, studies suggest that TMP resistant *E. coli* does not confer resistance to TMP-SMX (Murray et al., 1985). This is demonstrated by the 15 isolates which remained susceptible to the synergistic effect of the combination displayed by the antibiotic disk during AST (Bushby &

Hitchings, 1968). Auckland Hospital did not test *E. coli* against TMP-SMX and ESBL *E. coli* against TMP-SMX and amoxicillin-clavulanic acid, therefore it is unclear where the results obtained in the present study trend against Auckland data (LabPLUS, 2023). There is available data which only reports susceptibility to TMP (Heffernan et al., 2009; LabPLUS, 2023; Ussher et al., 2020). To our knowledge, no published or publicly available data which report the susceptibility of clinical UPEC isolates to TMP-SMX. It was concluded that many of their isolates displayed multi-resistance to three or more antibiotic classes, where 65.5% of these *E. coli* isolates were resistant to at least ciprofloxacin, aminoglycosides, and TMP. ESBL-positive *E. coli* demonstrated 72.7% to TMP. Although sample sizes varied in these studies it is suggested that the occurrence of TMP resistant *E. coli* is rising in NZ (Heffernan et al., 2009). This is supported by Mangin et al. (2005) where it was concluded that all tested uropathogens in their study displayed an increased resistance rate to TMP over a two year period. However, this study mentioned that their sample numbers were too small to determine if there was a statistically significant trend displayed by *E. coli* or other studied uropathogens. These two studies provide the most recent data involving TMP resistance of uropathogens in a selected NZ population. This highlights the importance of the findings of this chapter investigating recent TMP resistance of UPEC. Global TMP-SMX resistance in UTI associated *E. coli* has risen from < 10% in the 1990s to over 30% by the early 2010s (Critchley et al., 2019). Comparison of susceptibility from AUT results in Table 2.4 with the susceptibility percentages from Table 2.1, showed values matched currently available NZ surveillance data. Both AUT and Auckland Hospital *E. coli* susceptibility data showed ≥ 90% susceptible to meropenem and < 70% susceptibility to TMP. There were differences in percentage susceptibility values for amoxicillin-clavulanic acid and nitrofurantoin.

Uncomplicated UTI treatment is generally empiric which follows local resistance epidemiology and susceptibility patterns (Frisbie et al., 2022). Guidelines advise using an antimicrobial when local *E. coli* resistance exceeds 20%. Therefore TMP-SMX is used in regions where *E. coli* susceptibility is ≥80% (D. S. Lee et al., 2018b; Novelli & Rosi, 2017). Most of Southern Europe, fluoroquinolones and TMP-SMX resistance are above 20%, so they are not used for empirical treatment (D. S. Lee et al., 2018a; Novelli & Rosi, 2017). Combination therapy may slow bacterial resistance, however TMP-SMX resistance still emerges via molecular means which will be explored further in Chapter 3 (Darby et al., 2022).

Resistance has declined in some regions where reduced antibiotic prescriptions was noted, however this has not been observed by TMP-SMX in areas such as Sweden and the United Kingdom (D. S. Lee et al., 2018a). Antibiotic exposure extends beyond therapeutic use in humans, therefore antibiotic selection pressures may not be the only driving force of resistance (Christaki et al., 2020). MDR is a cause of concern in clinical applications, especially as TMP may

prescribed as TMP-SMX. The widespread use of TMP-SMX has resulted in a progressive emergence of resistance strains (Eliopoulos & Huovinen, 2001). These rates have been reported as high as 20% in several European countries and in the United States, and even in some developing countries (Gupta et al., 2011; Kot, 2019). This has limited the modern management of UTIs stated by clinical guidelines. Several studies have suggested that patient UTIs associated with TMP-SMX resistant pathogens are worse than those with solely UTI susceptible isolates (Novelli & Rosi, 2017).

If time permitted, secondary testing following disk diffusion assays could have been performed, where the isolates which exhibited resistance to the TMP (5 µg) disk may have been further interpreted using E-test gradient AST, as seen in Figure 1.9. This would have provided quantitative data to guide the selection of TMP concentrations during MIC studies (Baker et al., 1991; Joyce et al., 1992; Wiegand et al., 2008). Five isolates were susceptible to TMP suggesting resistance was lost during their transportation from Middlemore Hospital potentially due to freeze-thaw effects (Ray & Speck, 1973).

The MDR results of isolates in Table 2.4 showed that many of the isolates were only resistant to one or two drug classes. Understanding phenotypic MDR seen during clinical AST requires investigating genotypic mechanisms involved with genes and their expression (Hattab et al., 2024; Novelli & Bolla, 2024). Molecular resistance is investigated in Chapter 3.

2.4.2 Determination of Resistance Levels and Confirmation of Isolates

To assess the phenotypic resistance of isolates at TMP concentrations above the clinical breakpoint of 32 µg/mL, MICs were determined using OD curves. Results obtained in this study showed that whilst the 74 isolates may be interpreted as being TMP resistant via disk diffusion assays, they demonstrate different growth rates and characteristics over the 24-hour period at high TMP concentrations. Furthermore, the three levels of resistance showed differing numbers within low, medium, and high levels of resistance. Growth curves obtained from the OD may be used to further interpret the phenotypic TMP resistance of each isolate by revealing differences in growth rate, lag phase, and bacterial populations in the presence of TMP (Chandrasekaran & Jiang, 2019).

Resistance levels were determined using two methods, where both provided a criterion for grouping isolates from disk diffusion assay AST into different levels of TMP resistance. The first was based on visible growth curve features where isolates demonstrating similar OD values at the tested TMP concentrations were grouped together. The second based on the estimated MIC value of the isolates. As mentioned in Section 2.3.2.1 and Table 2.6, all these isolates

demonstrated MICs above 32 µg/mL, and suggested resistant isolates were able to tolerate a TMP concentration of upwards of 128 times above the clinical breakpoint as demonstrated by growth curves. This is expected as all 74 isolates presented TMP resistance during disk diffusion assay testing where the clinical breakpoint is 32 µg/mL (CLSI, 2023). Determining MIC values above the clinical breakpoint may provide a quantitative measure of resistance levels to identify extreme phenotypes (Kowalska-Krochmal & Dudek-Wicher, 2021). This may be necessary when monitoring epidemiological trends and shifts in MIC distributions in bacterial populations where resistance determinants can spread by molecular means (Catalán et al., 2022; Witzany et al., 2023). This may direct patient treatment plans, such as empirical treatment (as discussed in Section 1.3.4.4) as this relies on geographic epidemiology (Pujades-Rodriguez et al., 2019)

As mentioned in Section 2.1 the use of phenotypic observations from OD growth curves and their statistical interpretation during MIC AST provides a quantitative approach, as specific cut offs may be used for determining resistance thresholds (Axelsson et al., 2024). As presented in Figure 2.7 and Figure 2.8, isolates classified as resistant during disk assay diffusion demonstrate different growth curves during MIC testing. These differences can influence how growth rates are interpreted when assessing isolate tolerance at specific TMP concentrations, consequently introducing variability in the cut off features and OD values which are used to categorise resistance levels. Studies have used thresholds to determine assay performance of bacterial cultures during microplate readings (Vinchhi et al., 2023). Bacterial cultures which acted as growth controls (no antibiotics present) had to grow above a threshold after the first 90 minutes of incubation. These studies have found that the growth threshold would increase in absorbance of ≥ 50% (Axelsson et al., 2020; Axelsson et al., 2024; Jung et al., 2016; Lange et al., 2014; Maxson et al., 2017; Sparbier et al., 2016). To the author's knowledge, the use of a criterium for the classification of low, medium, and high resistant isolates has not been investigated in TMP resistance in similar research, especially at a national level.

Studies have generated two different approaches to statistically generate models and criteria to compare the susceptibility of their respective organisms to antibiotics (Bretonnière et al., 2016; Grazian, 2023). This statistical approach may have been used in this study to reduce bias when generating criteria in Section 2.2.3.1 and to allow comparison of isolates within TMP resistance levels The OD measurements over the 24 hours may have been used to statistically interpreted generating a dose-response curve using the normalised growth rate as a function of the TMP concentration (Angermayr et al., 2022). Computational tools such as the *Growthcurver* package offered by R v4.4.0 via R-studio may provide a more reliable and reproducible approach to interpret growth data compared to visually estimating MIC values (Sprouffske & Wagner, 2016). This statistical modelling of resistance breakpoints may be used to detect subtle resistance

phenotypes which may be overlooked by manual or visual interpretation (Catalán et al., 2022; Kowalska-Krochmal & Dudek-Wicher, 2021).

Most AMR studies rely on clinical breakpoints to classify isolates into categories of susceptible or resistant, therefore the extent of resistance above this threshold may be overlooked (Kowalska-Krochmal & Dudek-Wicher, 2021). Examining *E. coli* tolerance to TMP well above the clinical breakpoint of 32 µg/mL is novel as it allows for differentiation between low and high resistance phenotypes which can be further investigated for contributing molecular determinants (Blair et al., 2015; Catalán et al., 2022; CLSI, 2023).

2.4.3 Conclusion

This chapter aimed to characterise *E. coli* isolates resistant to TMP from UTI patients at Middlemore Hospital. Resistance was confirmed using disk diffusion assay AST, followed by MIC interpretation using OD curves to generate a TMP resistance level criterion. During disk assay AST of the 106 isolates obtained from Middlemore Hospital, three were non-viable, 98 demonstrated resistance to TMP and 83 were also resistant to the combination TMP-SMX. Resistance to other antibiotics included one isolate resistant to meropenem, two to nitrofurantoin, and eight to amoxicillin-clavulanic acid. Based on these results, isolates were categorised into low, medium, and high levels of TMP resistance, with further investigations conducted in Chapter 3.

Chapter 3 Whole Genome Sequencing of *E. coli*

3.1 Introduction

Phenotypic methods involve antibiotic susceptibility testing (AST) such as disk diffusion assays and minimum inhibitory concentration (MIC) testing, offer limited information on gene resistance epidemiology (Boolchandani et al., 2019). Therefore, whole genome sequencing (WGS) is a common tool which is used to identify potential novel genes and resistance mechanisms from genetic data (Ellington et al., 2017). Large accessory genomes may be found in *E. coli* isolates where specific uropathogenic genes may be found, however genes commonly associated with uropathogenic *Escherichia coli* (UPEC) pathogenesis are not well established (Shea et al., 2022). Therefore, the construction of whole genomes of resistant isolates are needed to establish the genes and mechanisms associated with trimethoprim (TMP) resistance. Sequencing the whole bacterial genome can provide a snapshot of an abundant information at once. This can be used to identify and type pathogens, analyse their genetic relationships, horizontal gene transfer between pathogens, and detect antimicrobial resistance (AMR) genes (Kim et al., 2023). Bacteria are capable of multiple biochemical pathways to develop antibiotic resistance and adopt these pathways to resist the effects of these antibiotics for survival (Sultan et al., 2018). As mentioned in Section 1.3.5.1, the methods of AMR mechanisms exhibited by bacteria include antibiotic inactivation, target site modification (replacement, protection or alteration), active efflux, and limiting antibiotic uptake by changing permeability (Galgano et al., 2025; Salam et al., 2023; Wanda, 2018). To investigate this, WGS can be used to determine the molecular distribution of AMR genes, transposable genetic elements (e.g., plasmids, transposons, and replicons) and the relationship with sequence types (Mohapatra et al., 2023).

There are over 4,000 genes present in *E. coli* with studies identifying a set of approximately 100-300 genes influencing bacterial sensitivity to multiple antibiotics (Otoupal et al., 2021). Evolution of variants are a result of gene acquisition events and loss of genetic information due to deoxyribonucleic acid (DNA) rearrangements and point mutations. Therefore, the *E. coli* genome consists of constantly evolving DNA regions (Dobrindt et al., 2010). UPEC present extra virulence genes which are often strain-specific pathogenicity islands of clusters of virulence related genes and vary in content and genomic location (Bielecki et al., 2014). The genes associated with antibiotic survival suggest that there is a regulatory network to combat antibiotic stress therefore understanding these networks is important (Deter et al., 2021). Gram-negative bacteria such as *E. coli* and *Klebsiella* spp. are the most predominant uropathogens which have been isolated exhibiting high levels if TMP resistance (Somorin et al., 2022). In order to become

resistant to TMP, *E. coli* strains have spontaneous mutations in the *folA* promotor region, resulting in increases of FolA expression (Kordus & Baughn, 2019). TMP reduces the available THF by inhibiting DHFR which is encoded by the *folA* and *folM* genes in bacteria such as *E. coli* (Figure 1.5) (Kordus & Baughn, 2019; Sangurdekar et al., 2011). TMP chromosomal resistance may be the result of mutations which increase transcription of the *folA* gene which encodes DHFR, or from mutations which decrease the affinity of DHFR for TMP (Kordus & Baughn, 2019; Sánchez-Osuna et al., 2020). Evolution of TMP resistance in *E. coli* was due to the stepwise gain of resistance mutations in the *folA* gene which encodes for the DHFR gene (Manna et al., 2021). Based on recent available whole genome sequences from gram-negative bacteria several new TMP resistant genes encoding a FolA homologue have been found with some showing to experimentally confer TMP resistance (Ambrose & Hall, 2021a).

The WGS approach requires analysis tools such as Bandage to visualise assemblies, which can make it easier to identify problematic areas of the assembled genome. This may be useful for when assemblies contain many short contigs (Wick et al., 2015). The Comprehensive Antibiotic Resistance Database (CARD) which is a knowledgebase on the molecular elements of antibiotic resistance by using bioinformatics and information about antibiotics resistance genes, gene products, and resistance mechanisms (Alcock et al., 2022). Basic Local Alignment Search (BLAST) may also be used as a search tool for the known resistance genes and associated mutations in previously studies isolates where the overlapping of sequences may allow for isolate specific resistomes to be mapped (Su et al., 2019; van Belkum et al., 2020).

Orthologues are homologous genes which encode proteins in equivalent biological functions which originate from a common ancestral gene and are known as orthologous protein cluster (OPC) (Gabaldón & Koonin, 2013). Comparative genomics which identifies and characterises orthologues allows for the identification of similarities and differences for constructing species phylogenies (Altenhoff & Dessimoz, 2009; Gabaldón & Koonin, 2013). OrthoVenn3 (Sun et al., 2023) is a tool which may be used to investigate orthologous genes in strains. As mentioned in Section 1.3.3.2 using antifolate target mutation and variations in *dfrA* genes have been detected in TMP resistant isolates (Brolund et al., 2010; Grape et al., 2007; Somorin et al., 2022). Some of these variants and their locations have been compiled in Table 3.1 (Ambrose & Hall, 2021a).

Table 3.1: Examples of *dfrA* variant genes identified in *E. coli* TMP resistant isolates.

Variants	Location(s)	NCBI accession	Reference
dfrA1	Transposon and plasmids	CAA25445.1	Fling and Richards (1983)
dfrA5	Class 1 integron cassette on plasmids	CAA31356.1	Sundström et al. (1988)
dfrA7	Class 1 integron cassette on plasmids	CAA41326.1	Sundström et al. (1993)
dfrA12	Class 1 integron cassette on plasmids	CAA79767.1	Heikkilä et al. (1993)
dfrA14	Plasmid-borne cassette	CAA90688.1	Young et al. (1994)
dfrA17	Class 1 integron cassette on plasmids	AAD50825.1	White et al. (2000)

As mentioned in Chapter 2, studies have investigated the relationship between low and high-level antibiotic resistance as defined by the antibiotic MIC, whereby mutations in genes may provide opportunities for low antibiotic resistance levels (Baquero, 2001; Baquero & Blázquez, 1997). Research has explored TMP resistance mechanisms and potential genes in *E. coli* strains isolated from clinical, environmental, food, and even murine models (Brolund et al., 2010; Grey et al., 1979; Jakovljev et al., 2022; Kordus & Baughn, 2019; Schreiber et al., 2017; Shea et al., 2022). However, these gene sets and their influence in human uropathogenesis have not been fully understood (Schreiber et al., 2017; Shea et al., 2022; Subashchandrabose et al., 2014).

Although international studies have investigated TMP resistance in UPEC using WGS and comparative genomics, to the best of our knowledge this has not been done in NZ clinical UPEC strains. Therefore, to investigate this, isolates from Chapter 2 were analysed for TMP resistance mechanisms and the associated genes of interest. This has been outlined in Figure 3.1 and further described in Figure 3.2.

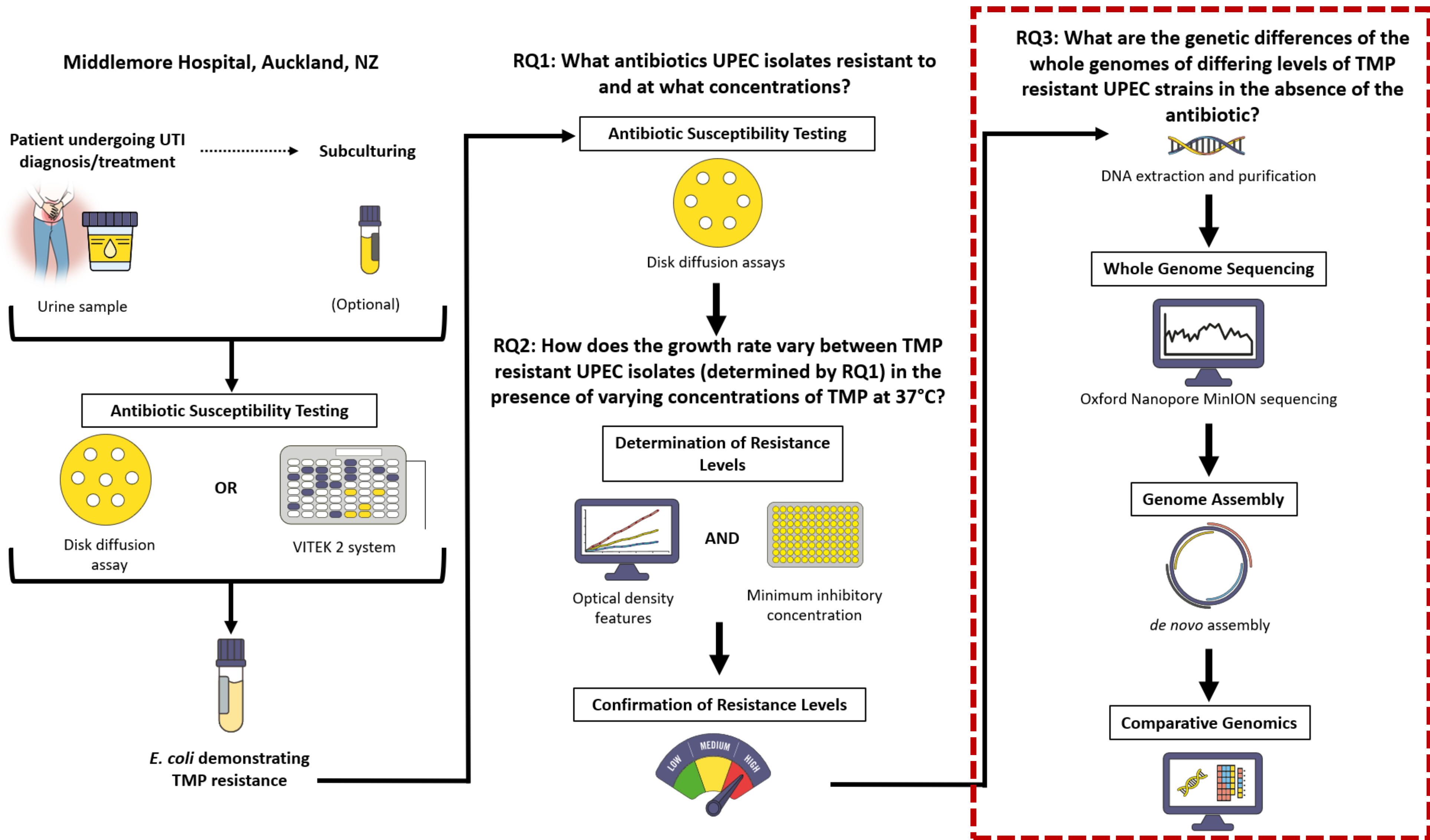

Figure 3.1: Schematic of the RQs of this study. Chapter 3 RQ and aims are highlighted in red.

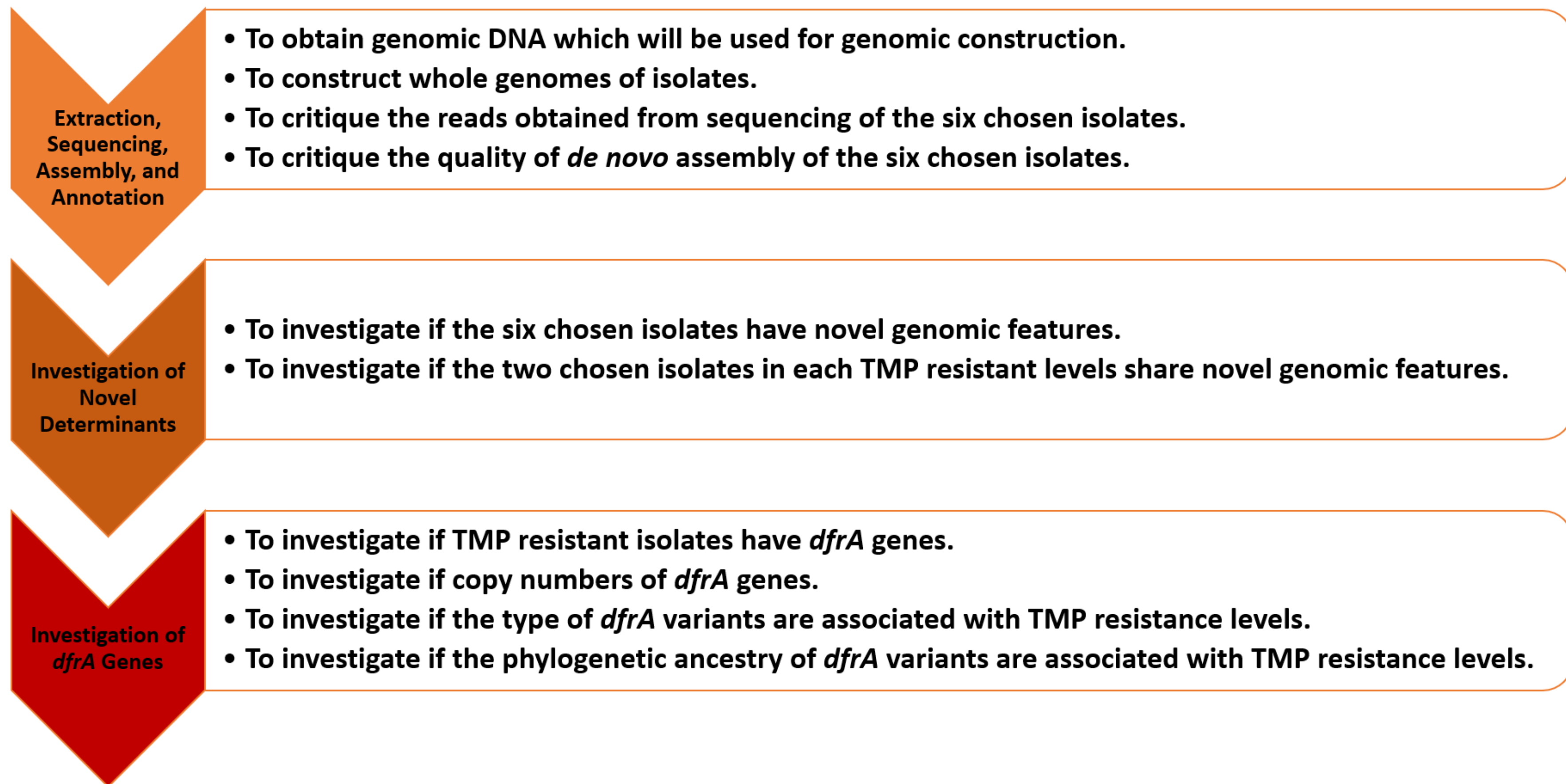

Figure 3.2: Chapter 3 method objectives.

3.2 Methods and Materials

3.2.1 Genomic DNA Extraction

The four isolates for each resistance level (i.e., low, medium, high) from Chapter 2 were used for genomic DNA extractions. Low resistance isolates were 25, 29, 45, and 98, medium resistance isolates were 01, 43, 72, and 79, and high resistance isolates were 23, 20, 21, and 57. These isolates were chosen from each resistance level as they met both criterium of optical density (OD) features (Table 2.5) and estimated MIC (Table 2.6) for their respective resistance level.

3.2.1.1 Commercial Kit Extraction

Overnight cultures were prepared as stated in Section 2.2 for the isolates listed in Section 3.2.1. Isolate 21 did not have turbidity in overnight cultures and was not used for further testing. The extraction of isolate genomic deoxyribonucleic acid (gDNA) was obtained via the commercial extraction kit DNEasy Blood and Tissue Mini Kit (Qiagen, Hilden, Germany) following the manufacture's protocol.

3.2.2 Quality and Quantity of gDNA Extractions

To determine the quality of the extracted gDNA from Section 3.2.1 an agarose gel electrophoresis was run. Each lane was loaded with 5 µL of gDNA and 1 µL SYBER Safe DNA Gel Stain (ThermoFisher Scientific, Massachusetts, USA) alongside 0.1 µg/µL 100 bp DNA ladder (Solis BioDyne, Estonia). The last lane was run as a no template control (NTC). The gel was viewed and photographed under ultraviolet light with an AlphaImager® HP (Alpha Innotech, California, USA).

3.2.3 AMpure XP Bead Purification

AMPure XP Beads (Beckman Coulter, Brea, CA) were used prior to library preparation to remove any contaminants before sequencing. The purification and hybridisation were performed as stated in the manufacturers protocol (Fisher et al., 2022).

3.2.4 Oxford Nanopore Minion Library Preparation and Genome Sequencing

The two isolates from each resistance level which showed the best quality and quantity of extracted gDNA from the steps in Section 3.2.2 and showed sterility in negative control wells in Figure 2.9 were then used for further analysis. Low resistance isolates were 25 and 98, medium resistance isolates were 01 and 79, and high resistance isolates were 23 and 57.

In order to prepare the sequencing library, the Rapid Sequencing Kit (SQK-RAD14) (Oxford Nanopore, Oxford, UK) was used. Purified 10 µL of gDNA from isolates 01, 23, 25, 57, 79, and 98 were each transferred into 1.5 mL LoBind tubes containing 1 µL fragmentation mix and gently agitated. Tubes were incubated for 2 minutes at 30 °C followed by 2 minutes at 80 °C then cooled rapidly using a cooling block. Rapid adapter buffer solution was prepared by diluting 1.5 µL rapid adapter with 3.5 µL adapter buffer, 1 µL of the rapid adapter buffer solution was transferred into each 1.5 mL LoBind tube containing isolate gDNA. The DNA library was prepared with 12 µL of the rapid adapter buffer solution and DNA was transferred into a transfer tube containing 37.5 µL of sequencing buffer and 25.5 µL mixed library bead solution and mixed. Nanopore priming mix was prepared by mixing 1.17 mL flow cell flush, 5 µL bovine serum albumin, and 30 µL flow cell tether. The Nanopore MinION flongle (Oxford Nanopore, Oxford, UK) was then loaded with 200 µL of the Nanopore priming mix and 75 µL of DNA library. The flongle was then run for 24 hours under the default settings.

3.2.5 Genome Assembly

De novo genome assembly was completed in-house by assembling filtered reads into contigs using SPAdes v3.15.5 (Bankevich et al., 2012) and Unicycler v0.5.0 (Wick et al., 2017) using the default settings.

3.2.6 Whole Genome Analysis

Assembled genomes were visualised by uploading the *.gfa files into Bandage v0.9.0 (Wick et al., 2015). After visualisation, circular contigs presumed to be plasmids were run through NCBI BLASTn suite (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using the default setting to confirm their identity to known *E. coli* plasmids.

Annotation of assembled isolate genomes was interpreted using RAST v2.0 (Aziz et al., 2008). The whole genome fasta files of each isolate was uploaded onto the RAST server (https://rast.nmpdr.org/) using the default settings.

3.2.7 Identification of Potential Novel Antibiotic Resistance Determinants

The orthologous clusters were identified using OrthoVenn3 (Sun et al., 2023). The amino acid *.faa files for each isolate were obtained from RAST, and the *.faa file of the susceptible strain *Escherichia coli* ATCC 25922 were uploaded into the OrthoVenn3 web interface (https://orthovenn3.bioinfotoolkits.net/home) using the default settings using the OrthoMCL algorithm and an E-value $1x10^{-2}$, inflation value of 1.50, and annotation, protein similarity, and cluster relationship network enabled.

Shared clusters of interest were identified between the susceptible strain and all six TMP resistant isolates, as well as within each resistance level (Figure 3.3). The shared orthologous clusters related to AMR and pathogenicity were manually curated and searched through NCBI BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and InterPro (https://www.ebi.ac.uk/interpro/).

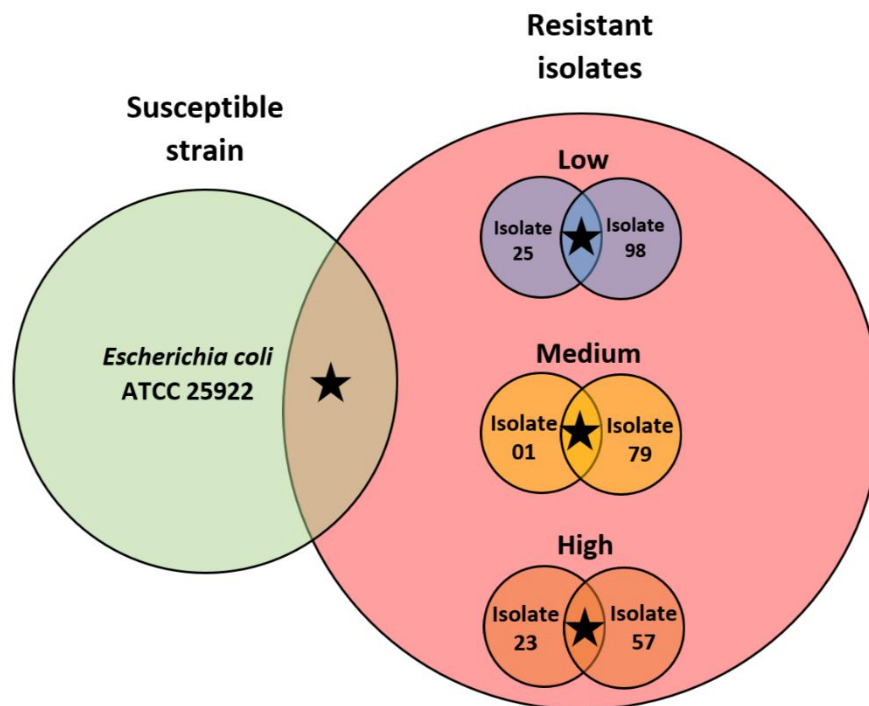

Figure 3.3: Schematic of clusters of interest from generated OrthoVenn3 Venn diagram.

Potential novel antibiotic genes were identified using CARD (McArthur et al., 2013) through their online tool (https://card.mcmaster.ca/analyze/rgi). Percentages for perfect and strict hits were identified for resistance mechanisms for each isolate. A heatmap of genes of interest was generated from the percentage hits from the CARD knowledge base.

3.2.8 Investigation of *dfrA* Genes

The presence of proteins associated in the folate pathway were identified using the annotated SEED genome browser. The nucleotide sequences of the *dfrA* genes identified by RAST from each isolate were verified using NCBI BLASTn. Nucleotide and protein maximum likelihood phylogenetic trees of the verified *dfrA* genes from each isolate were generated using the bootstrap method using 500 replications using MEGA11 (Tamura et al., 2021). *Salmonella enterica* serovar Typhi was used as an outgroup as it is also part of the Enterobacteriaceae group and to allow for rooting for phylogenetic trees.

3.3 Results

3.3.1 Genomic DNA Extraction

Isolate 21 from the high resistance level did not produce turbidity during overnight cultures and was excluded from further testing. The agarose gel showed solid bands at the expected location against the ladder (Figure 3.4). Isolate 45 showed a faint band above the 10,000 bp mark. The control lane showed no bands suggesting no contamination was present during the gDNA extraction procedure.

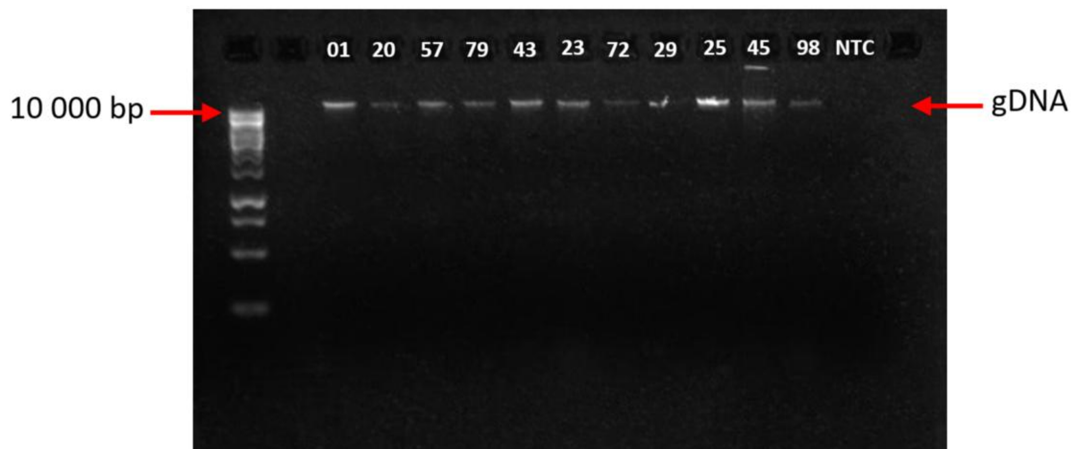

Figure 3.4: 1% agarose gel of isolates gDNA using the Qiagen DNeasy Mini Blood and Tissue Kit. NTC = no template control used as a negative control.

3.3.2 Whole Genome Analysis

Bandage (Wick et al., 2015) was used to visualise *de novo* assemblies of the six chosen isolates where circular contigs were seen for isolates 98, 01, 79, 23, and 57 (Figure 3.5). All isolates except isolate 25 showed a large circular contig with smaller contigs. The smaller contigs were run through BLASTn and showed to be part of *E. coli* genomes (results not shown). The generated visualisations suggested the presence of one or multiple plasmids for each isolate. A BLASTn search was done of the suspected plasmids and showed high similarity with other known *E. coli* plasmids (Table 3.2).

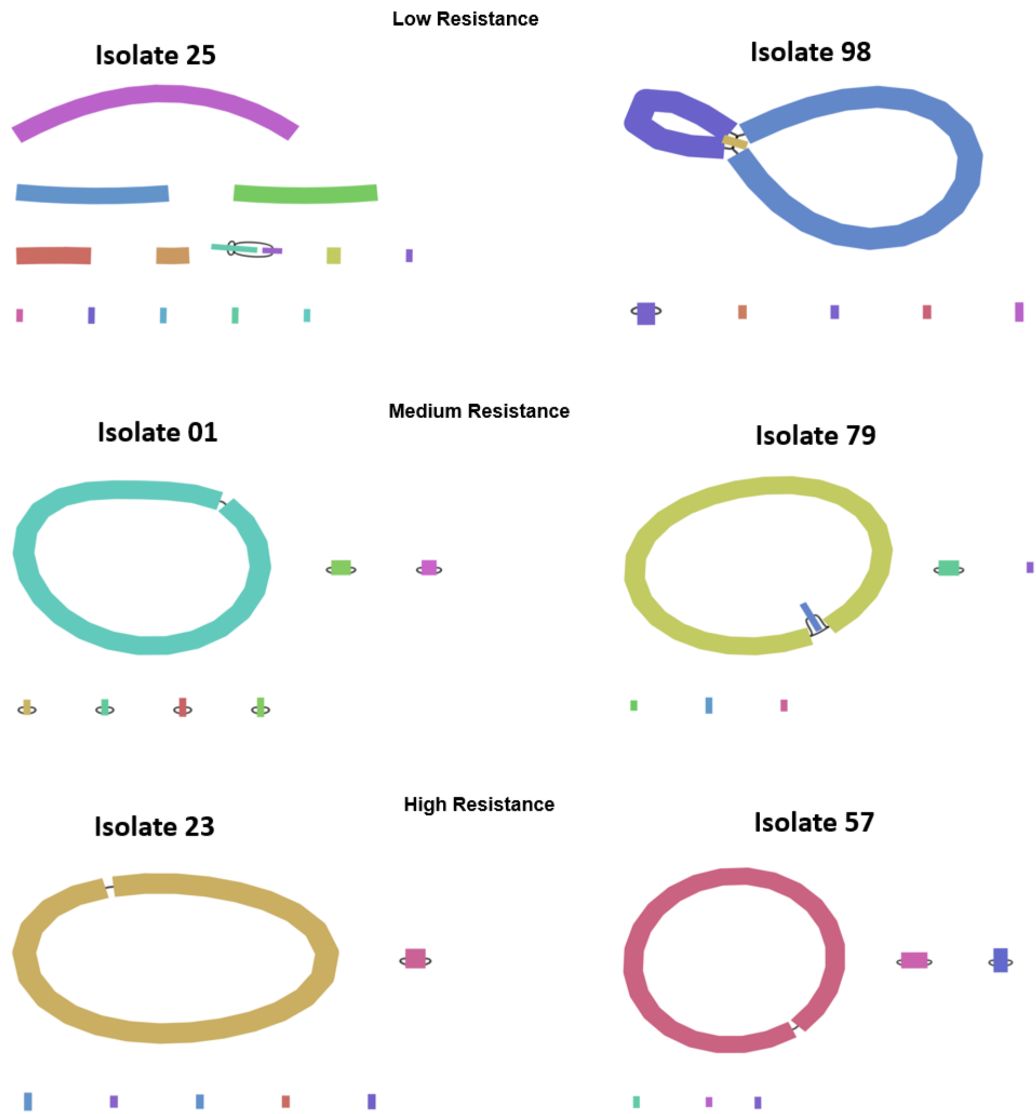

Figure 3.5: Assemblies of sequenced genomes as visualised by Bandage.

Results in Table 3.2 show BLAST analysis of TMP resistant UPEC isolates and predicted plasmids as visualised by Bandage (Figure 3.5). The total number of plasmids were lower in the high TMP resistance group than the medium and low. Isolates in the low resistance level showed high query coverage and sequency identity to the recorded *E. coli* plasmids in the database. Medium resistance overall had larger bp plasmids. Isolates in the high resistance level show complete identity and full query coverage.

Table 3.2: BLASTn results of Bandage plasmids from each isolate.

TMP Resistance Level	Isolate	Query length (bp)	Description	Query cover	E-value	Percentage (%) identity	Accession
Low	25	27,219	*Escherichia coli* strain GN02461 plasmid p2461-1	100%	0.0	99.17%	CP095535.1
		24,829	*Escherichia coli* strain 19KM1766N plasmid p19KM1766_1	100%	0.0	99.40%	CP151006.1
		13,794	*Escherichia coli* strain OXEC-422 plasmid unnamed	92%	0.0	96.07%	CP164549.1
		508	*Escherichia coli* strain MS20673 plasmid pMS20673C	99%	5e-151	96.18%	CP082070.1
	98	113,069	*Escherichia coli* strain GN02350 plasmid p2350-1	100%	0.0	100%	CP095519.1
Medium	01	140,023	*Escherichia coli* strain D6 plasmid A	93%	0.0	99.95%	CP010149.1
		107,501	*Escherichia coli* strain MRSN346638 plasmid pMRSN346638_119.3	89%	0.0	98.75%	CP018116.1
		41,784	*Escherichia coli* plasmid pEC14_35	73%	0.0	97.91%	JN935899.1
		19,938	*Escherichia coli* strain C41 plasmid pC4102	100%	0.0	99.97%	CP127253.1
		12,580	*Escherichia coli* strain G3/10 plasmid pSYM2	100%	0.0	99.95%	CP060078.1
	79	149,419	*Escherichia coli* strain EW658 plasmid pEW658-TEM	100%	0.0	99.99%	OM735810.1
High	23	131,531	*Escherichia coli* strain E17EC0423 plasmid pE17EC0423-1	100%	0.0	99.97%	CP088462.1
		7,199	*Escherichia coli* strain CRE287 plasmid pKP287-2	100%	0.0	100%	CP166756.1
	57	111,012	*Escherichia coli* strain LH09-a plasmid pLH09-a-B	98%	0.0	99.99%	CP100546.1

General genomic features of *E. coli* genomes are presented in Table 3.3. The genome sizes were approximately 4.9-5.3 kbp, with GC content 50%. Total genes were approximately 5,000 for each isolate where 94-97% were protein coding.

Table 3.3: Genomic features of draft genomes of isolates from RAST.

	Low		Medium		High	
Features	**25**	**98**	**01**	**79**	**23**	**57**
Genome size (bp)	4,862,063	5,317,632	5,074,333	4,976,830	5,067,256	5,144,201
GC content (%)	51.0	50.4	50.8	50.9	50.6	50.9
Number of contigs	13	5	7	2	3	3
Total genes	4,947	5,506	5,306	5,138	5,180	5,393
Protein encoding genes (% of total genes)	4,795 (96.93%)	5,282 (95.93%)	5,012 (94.46%)	4,866 (94.71%)	4,980 (96.14%)	5,074 (94.08%)
Hypothetical proteins (% of CDS)	366 (7.63%)	645 (12.21%)	527 (10.51%)	427 (8.78%)	508 (10.20%)	523 (10.31%)
Total RNA genes	108	111	105	109	109	109
rRNA genes (5S, 16S, 23S rRNA)	22(8, 7, 7)	22 (8, 7, 7)	22 (8, 7, 7)	22 (8, 7, 7)	22 (8, 7, 7)	22 (8, 7, 7)
tRNA genes	86	89	83	87	87	87

3.3.3 Identification of Potential Novel Antibiotic Resistance Determinants

3.3.3.1 OrthoVenn3

Analysis of OPCs (Figure 3.6) showed 3520 clusters among the tested isolates and the susceptible strain *Escherichia coli* ATCC 25922. All isolates showed notably higher cluster counts than the susceptible strain where isolate 57 showed the largest number of clusters compared to other isolates. The 152 shared clusters of resistant isolates are highlighted. Cluster occurrences may be found in Appendix D: OrthoVenn3 Gene Cluster Occurrence.

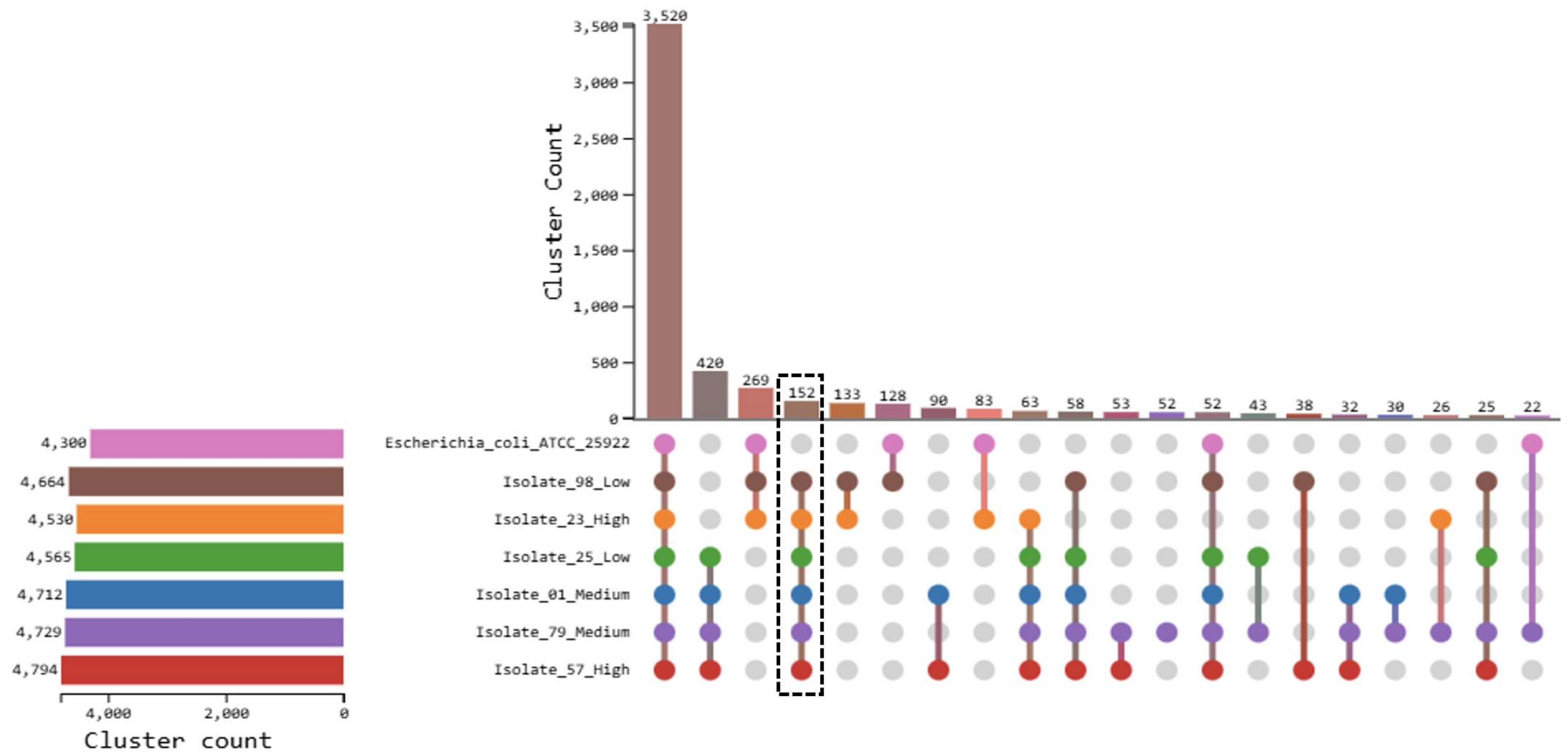

Figure 3.6: UpSet plot of orthologous clusters for isolates as predicted by OrthoVenn3.

The OrthoVenn3 cluster Venn diagram (Figure 3.7) shows 152 OPCs are shared among the six TMP resistant isolates. The biological processes clusters shared between the six TMP resistant isolates and the susceptible strain showed 32 shared processes. The largest category included metabolic processes, conjugation, and nitrogen compound metabolic processes. However, there were variations in the number of biological clusters between each TMP resistance level and which processes these were. Full results of cluster count for biological processes may be found in Appendix D: OrthoVenn3 Gene Cluster Occurrence (Table D2) as BLASTp and InterPro were used to validate AMR and pathogenic clusters. AMR functional clusters included plasmid transfer and metabolic pathways, with two clusters showing proteins associated with the folate pathway as mentioned in Section 1.3.4.3. Cluster 949 contained seven protein clusters which encode for the TMP resistant dihydrofolate reductase. Cluster 88 contained eight protein clusters which encode sulfonamide resistant dihydropteroate synthase Sul.

The two low TMP resistant isolates shared six OPCs, each containing two proteins. These clusters were associated with DNA mediated transposons (cluster 5120; P76071), β-glucan biosynthesis process (cluster 5240; B5YVS0), and the cell membrane (cluster 5385; P0AD04). Three additional shared clusters were identified without functional annotations.

The two medium TMP resistance isolates shared 30 OPCs, each containing two proteins. Several clusters were annotated with functional roles consisting of viral tail fibre assembly (cluster 5470; P0DJY5), viral tail assembly (clusters 5597; P51768, 5602; O64313, and 5615; O64312), viral capsid assembly (cluster 5631; P25476), DNA replication (cluster 5585; P21311), and DNA repair (cluster 5612; P21320). Other clusters included protein complex oligomerisation (cluster 5566; P21323), peptidoglycan catabolic process (cluster 5567; Q6XQ98), and zinc ion binding (cluster 5581; P41059). The remaining clusters were identified without functional annotations.

The two high TMP resistance isolates shared nine OPC with most of these clusters containing two proteins. These clusters included cytoplasm (cluster 5510; P64525), transketolase activity (cluster 5643; Q58092). Other clusters included plasmid maintenance (cluster 5656; Q52042) and DNA binding (cluster 5657; P62553). One cluster contained four proteins and were annotated for sequence specific DNA binding (cluster 4584; P19768). The remaining clusters were identified without functional annotations.

The two low and two medium TMP resistance isolates shared one OPC which contained four proteins and were identified without a functional annotation. The two low and two high TMP resistance isolates shared one OPC which contained five proteins and were identified without a functional annotation. The two medium and two high TMP resistance isolates shared three OPCs which each contained four proteins and were identified without functional annotations.

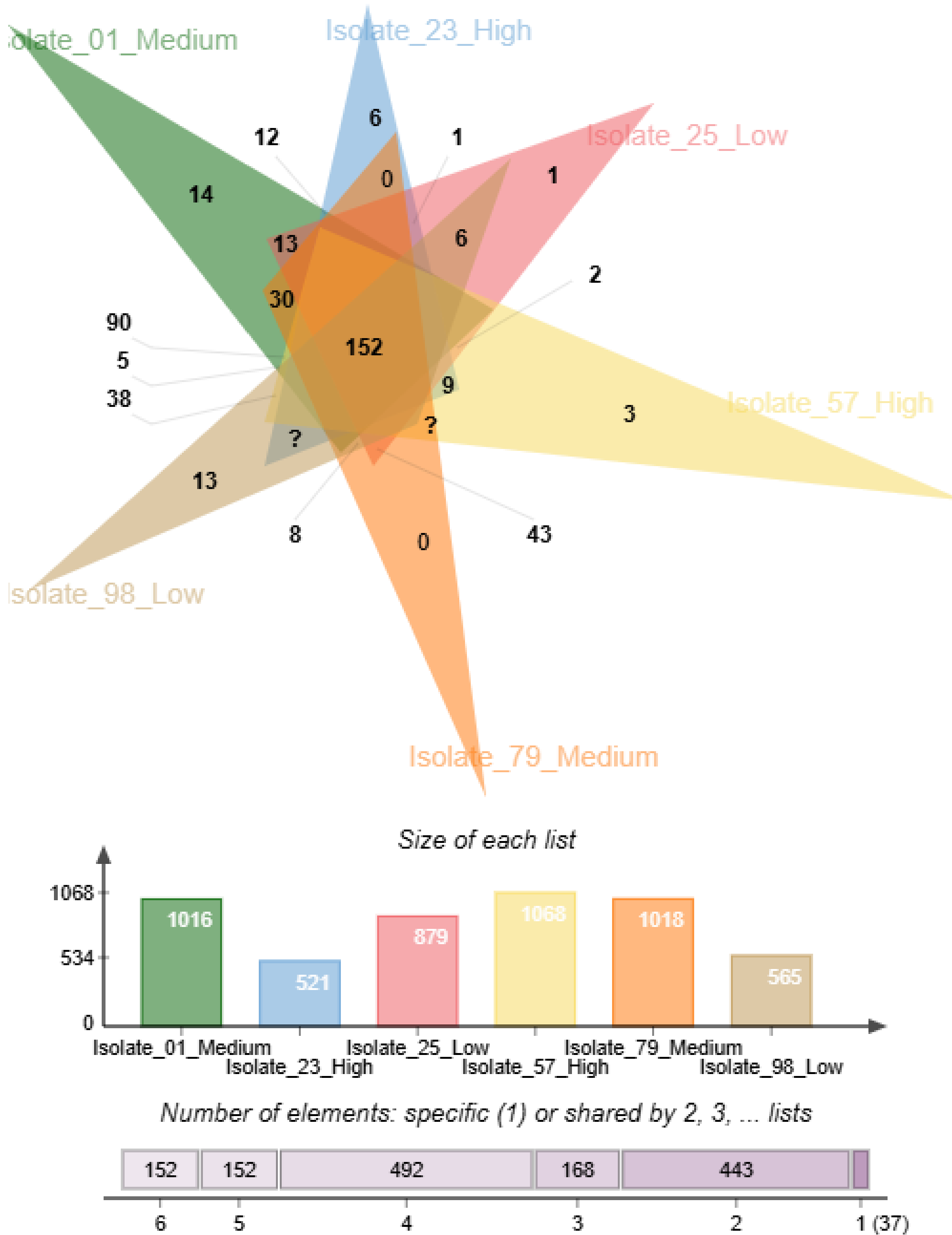

Figure 3.7: Venn diagram of clusters in resistant isolates produced by OrthoVenn3.

3.3.3.2 The Comprehensive Antibiotic Resistance Database

The Comprehensive Antibiotic Resistance Database (CARD) was used as a reference database to identify genetic determinants of known AMR mechanisms present in the isolates (Table 3.4). Antibiotic efflux showed the highest perfect and strict hit counts across isolates and resistance

levels. Antibiotic target protection and antibiotic target alteration showed the lowest hit counts across isolates and resistance levels. However, there were no observable trends among the three resistance levels for the six AMR mechanisms. Isolate 23 showed greater hits for perfect antibiotic inactivation hits than the other isolates presented.

Table 3.4: Antibiotic resistance mechanism CARD hits for each for each isolate.

Resistance Level	Isolate		Antibiotic Efflux	Antibiotic Inactivation	Antibiotic Target Replacement	Antibiotic Target Protection	Antibiotic Target Alteration	Reduced Permeability to Antibiotic
Low	25	Perfect	14	0	1	0	0	1
		Strict	29	2	1	0	13	1
	98	Perfect	7	1	1	0	0	0
		Strict	38	3	1	0	13	2
Medium	01	Perfect	14	2	3	1	0	0
		Strict	30	2	0	0	13	2
	79	Perfect	15	1	1	0	0	1
		Strict	29	3	1	0	13	1
High	23	Perfect	7	6	2	0	1	0
		Strict	42	2	1	0	14	2
	57	Perfect	14	1	3	0	0	1
		Strict	30	3	0	0	14	1

Percentage similarity according to CARD of selected genes are presented in Figure 3.8. There were high identity scores (>98%) observed for multiple efflux pump associated genes for the six resistant isolates and susceptible strain. Genes encoding alternative antibiotic targets, including TMP resistance *dfrA* variants and sulfonamide resistance genes (*sul1* and *sul2*) varied across the six isolates. The susceptible strain did not show any CARD results for the antibiotic target replacement genes *dfrA* and *sul*.

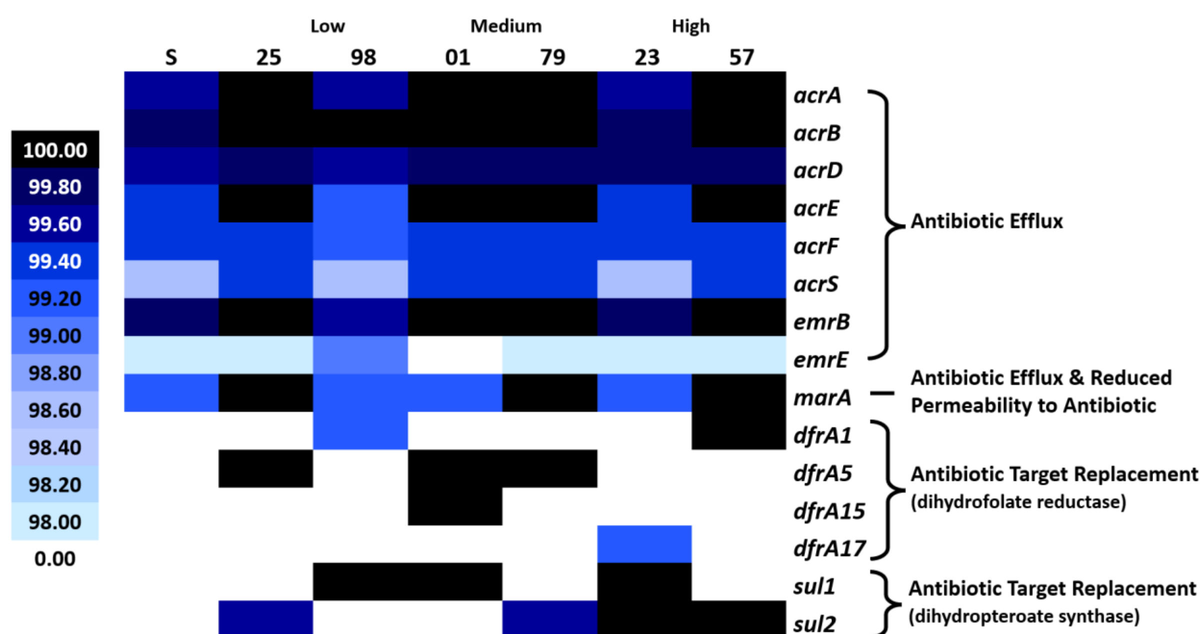

Figure 3.8: Heatmap showing percentage similarity according to CARD of selected genes. S denotes the susceptible strain *Escherichia coli* ATCC 25922.

3.3.3.3 Investigation of *dfrA* Genes

Isolates across all three resistance levels had *folM*, *folA*, and *dfrA* coding genes according to RAST (Table 3.5). RAST genome annotations identified multiple *dfrA* genes in each isolate, with gene copies varying between two and three; both low TMP resistant isolates contained two copies.

Table 3.5: Presence of antifolate genes in isolates and the number of copies according to RAST.

Resistance Level	Isolate	*folM*	*folA*	*dfrA*
Low	25	✓	✓	✓✓
	98	✓	✓	✓✓
Medium	01	✓	✓	✓✓✓
	79	✓	✓	✓✓
High	23	✓	✓	✓✓
	57	✓	✓	✓✓✓

Ticks denote the number of gene copies present.

RAST Dihydrofolate reductase (EC 1.5.1.3) function annotations for each isolate were validated using BLASTn (Table 3.6). Each isolate showed a RAST annotation was a BLASTn hit for *folA* genes. BLASTn showed the *dfrA* genes were present in each isolate with 100% identity and that the genes were located on plasmids.

Table 3.6: RAST and BLASTn results for *dfrA* genes from each isolate.

		RAST		BLASTn					
TMP Resistance Level	**Isolate**	**Contig**	**Query length (bp)**	**Description**	**Query cover**	**E-value**	**Percentage Identity**	**Accession**	**Gene**
Low	25	Contig 1	591	*Escherichia coli* strain 1585m1 chromosome	100%	0.0	100%	CP086391.1	*folA*
		Contig 13	474	*Escherichia coli* strain OXEC-517 plasmid p2	100%	0.0	100%	CP163646.1	*dfrA5*
	98	Contig 1	474	*Escherichia coli* strain C439 plasmid pT82A	100%	0.0	100%	CP046026.1	*dfrA1*
		Contig 2	591	*Escherichia coli* strain OXEC-19 chromosome	100%	0.0	100%	CP163780.1	*folA*
Medium	01	Contig 1	591	*Escherichia coli* isolate L5_E1779_ETEC genome assembly	100%	0.0	100%	LR883006.1	*folA*
		Contig 2	474	*Escherichia coli* strain CFS3313 plasmid pCFS3313-1	100%	0.0	100%	CP026940.2	*dfrA5*
		Contig 3	474	*Escherichia coli* strain EF7-18-51 plasmid pEF7-18-51_1	100%	0.0	100%	CP063488.1	*dfrA15*
	79	Contig 1	591	*Escherichia coli* strain 6A_B2017 chromosome	100%	0.0	100%	CP123274.1	*folA*
		Contig 7	474	*Escherichia coli* strain OXEC-544 plasmid p2	100%	0.0	100%	CP163847.1	*dfrA5*
High	23	Contig 1	591	*Escherichia coli* strain OXEC-506 chromosome	100%	0.0	100%	CP165203.1	*folA*
		Contig 2	474	*Escherichia coli* strain 675SK2 plasmid p675SK2_B	100%	0.0	100%	CP027703.1	*dfrA17*
	57	Contig 1	474	*Escherichia coli* strain 1448 plasmid p1448_1	100%	0.0	100%	CP184063.1	*dfrA1*
		Contig 3	480	*Escherichia coli* strain OXEC-132 chromosome	100%	0.0	100%	CP163888.1	*folA*

In order to investigate potential evolutionary relationships between *dfrA* variants in isolates of the same TMP resistance level, nucleotide and protein maximum likelihood phylogenetic trees were generated using MEGA11 (Tamura et al., 2021). The maximum likelihood phylogenetic tree constructed from isolate *dfrA* nucleotides (Figure 3.9) and proteins (Figure 3.10) showed bootstrap values ≥97%. The positions of *dfrA* gene variants in clades were the same for both trees. Low reseistance isolates (●), medium resistance (▲), and high resistance isolates (★) did not form clusters according to the resistance phenotype.

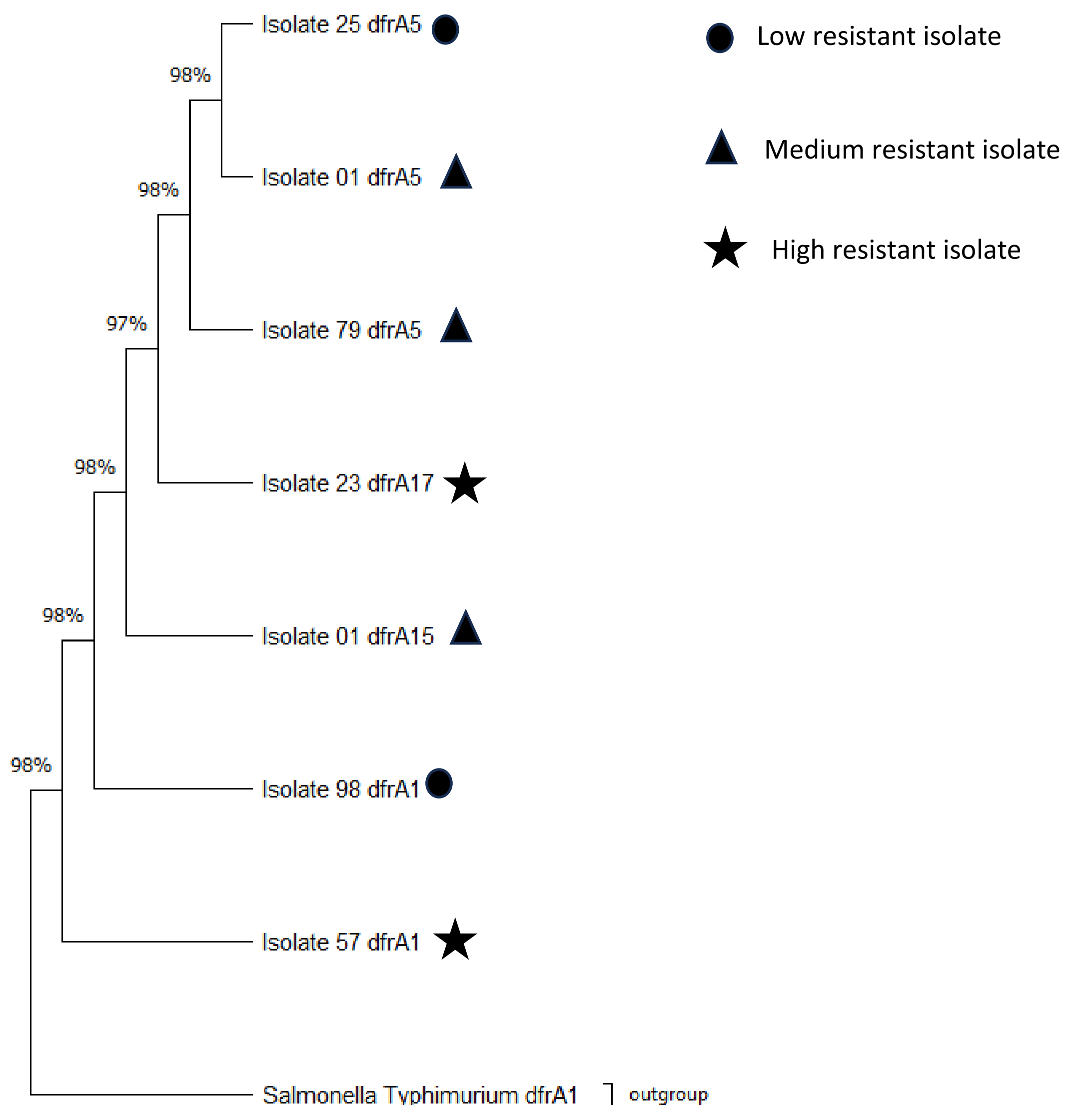

Figure 3.9: Nucleotide maximum likelihood phylogenetic tree of isolate *dfrA* variants.

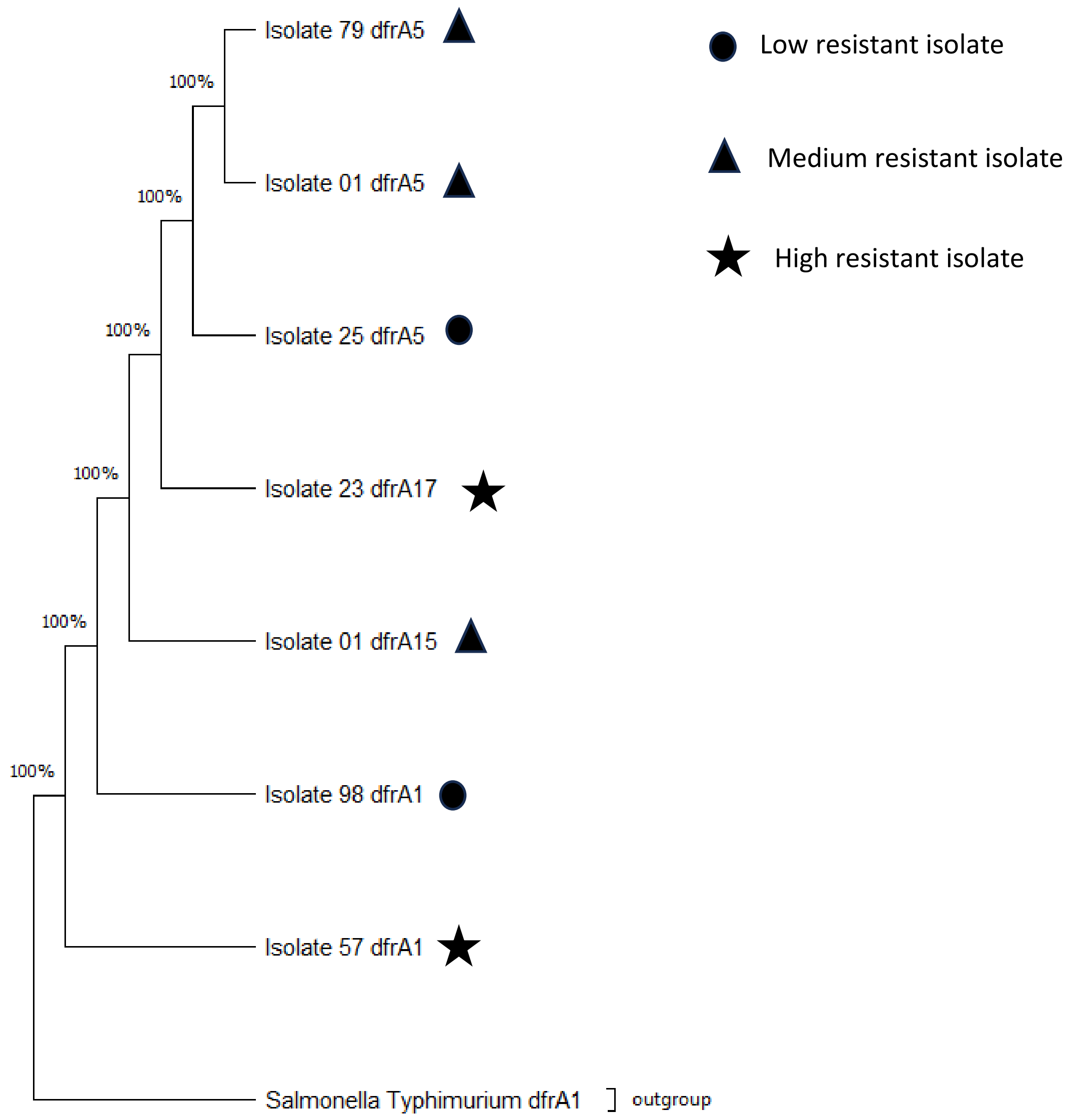

Figure 3.10: Protein maximum likelihood phylogenetic tree of isolate *dfrA* variants.

3.4 Discussion

In order to investigate the genetic elements underlying varying levels of TMP resistance, OrthoVenn3, RAST, and CARD were used. Studies involved in investigating antimicrobial resistance profiles have relied primarily on phenotypic analysis to differentiate strains into resistant, intermediate, and susceptible (CLSI, 2023; Matuschek et al., 2014; Ranjbar et al., 2020). To the author's knowledge, there have not been any previous research investigating the genetic elements which may influence the resistance tolerance to an antibiotic. Furthermore, the molecular basis of TMP resistance has not been explored within NZ clinical isolates.

3.4.1 Genomic DNA Extractions

To determine the quality of gDNA extracted from the DNEasy Blood and Tissue Mini Kit (Qiagen, Hilden, Germany) a 1% agarose gel was run. The thick bands at near the wells of the gel suggest large and complete gDNA was obtained during extraction (Arslan et al., 2021; Helling et al., 1974). Isolate 45 showed two bands of gDNA which may be a result of too much biomass transferred from the overnight culture prior to extraction (Xin et al., 2021).

3.4.2 Whole Genome Analysis

In order to assess the quality of the assembly of reads from MinIon (ONT) (Oxford, UK) sequencing, Bandage was used for genome visualisation (Wick et al., 2015). Five of the six isolates displayed one large circular contig alongside several smaller contigs, indicating that the *de novo* assemblies were generally successful and close to complete (Wick et al., 2017). These smaller contigs showed sequence similarity to other *E. coli* plasmids, via BLASTn analysis (Table 3.2), which are commonly found in *E. coli* genomes (Paganini et al., 2021). Plasmids cause a fitness cost on bacterial cells, suggesting their presence is the result of selective pressure, such as the pressure caused by antibiotics (Lee & Ko, 2021). Future directions could investigate whether the presence and the content of plasmids influence the level of TMP resistance, such as if high resistance isolates have more mobile elements than low resistance isolates (Paganini et al., 2021).

Results from RAST annotations (Table 3.3) showed genomes lengths of approximately 4.9-5.3 kbp and GC content of 50-51% which is characteristic of UPEC strains, as they typically have larger genome sizes than other *E. coli* strains (Lloyd et al., 2009; Subhadra et al., 2018).

3.4.3 Identification of Potential Novel Antibiotic Resistance Determinants

In order to identify potential novel antibiotic resistance determinants, OPCs and known resistance genes were compared within and between TMP resistance levels alongside known

resistance genes. The UpSet plot generated by OrthOvenn3 (Figure 3.6) showed 3520 OPCs shared among all resistant isolates and the susceptible strain, where clusters were identified to be housekeeping genes involved in replications, transcription, translation, and basic metabolic functions, as previously stated in Section 1.3.2.3 (Geurtsen et al., 2022; Leimbach et al., 2013; Rasko et al., 2008). The generated Venn diagram of the 152 OPCs shared between the six resistant isolates (Figure 3.7) were identified by BLASTp and InterPro to be variable accessory or unique genes which may have been acquired via horizontal gene transfer of plasmids and linked to adaptation, resistance, or virulence factors (Tantoso et al., 2022; Touchon et al., 2020). Clusters included folate pathway genes such as *dfrA* (cluster 949) and *sul* (cluster 88), suggests the importance these genes play in TMP and TMP-SMX resistance (Barraud & Ploy, 2015; Li et al., 2020).

Results from Figure 3.7 showed distinct clusters when interpreting relationships within TMP resistance levels (i.e., low, medium, and high). Low resistance isolates showed a cluster which is associated with DNA mediated transposons which may allow for antibiotic resistance genes to transfer across genomes and potentially species (Partridge et al., 2018; Stokes & Gillings, 2011). Cell membrane and wall clusters were also seen by low resistance isolates which can alter cell robustness to antibiotics (Choi & Lee, 2019; Delhaye et al., 2019; Morè et al., 2019). Medium resistance isolates showed viral assembly OPCs which may be the result of prophage activity and phage mediated horizontal gene transfer which can cause resistance traits (Touchon et al., 2017; Wendling et al., 2021). Clusters associated with DNA replication and repair were also seen within the medium level which may cause survival caused by antibiotic stress (Baharoglu & Mazel, 2014). Peptidoglycan catabolism clusters and envelope processes are linked to envelope remodelling which can affect structural integrity to antibiotics (Morè et al., 2019). Zinc binding and oligomerisation proteins can be associated in regulatory or enzymatic complexes relevant to antibiotic stress and resistance phenotypes (Sharma et al., 2017; Thota & Chubiz, 2019). High resistance OPCs showed plasmid maintenance which has been noted in bacteria with high tolerance to other antibiotics such as β-lactams and quinolones (Buckner et al., 2018; Lopatkin et al., 2017). The DNA binding and regulatory OPCs upregulate efflux and porins which has been seen in MDR strains (Sharma et al., 2017). The transketolase activity cluster has been shown through carbon metabolism studies to show relationships between metabolic states and antibiotic susceptibility and evolution to resistance (Zampieri et al., 2017). Investigating the influence of metabolism on AMR may be an area of interest for future research.

The low OPCs counts shared between the resistance groups (i.e., low and medium, low and high, medium and high) suggests resistance and degree of resistance may result from multiple mechanisms rather than a shared pathway (Baquero et al., 2021). The variation in cluster

numbers without functional annotations between different levels of TMP resistance may represent uncharacterised accessory genes which could contribute to different resistance phenotypes (Goldstone & Smith, 2017). This highlights the importance of using multiple databases to verify and characterise hypothetical proteins, as clusters with uncharacterised functional annotations in one database may show sequence similarities to AMR determinants in another database, therefore helping decipher the function of this annotated sequence (Nan et al., 2009).

All six isolates and susceptible strain showed differing percentage similarity to antibiotic efflux pumps and permeability genes in CARD (Figure 3.8). Differences in percentage similarities within TMP resistance levels may indicate allele variants or novel sequence divergence which are not currently represented in CARD (Lipworth et al., 2024). Further investigation should be done to determine if these are novel AMR determinants or if they are limited by the information offered in the database (Suarez & Martiny, 2024). The high percentage similarities seen with efflux pump genes is expected as these are important in resistance evolution as prolonged antibiotic exposure leads to increased efflux activity to reduce the drug concentrations in the cell (Viveiros et al., 2007). The differences in percentage similarities to the efflux regulatory gene *marA* may result from upregulation of AcrAB-TolC system which is vital in MDR and reducing drug permeability into bacterial cells (Sharma et al., 2017). Investigating this system may be an area of future work. The six TMP resistance isolates had a *dfrA* variant and a *sul* variant, which the susceptible strain did not which is expected of resistant isolates.

3.4.4 Investigation of *dfrA* Genes

RAST annotations in Table 3.4 showed the six isolates contain *folA* (chromosomal DHFR) and *folM*, which indicates the core folate pathway genes that are essential for THF dependent carbon metabolism as previously shown in Figure 1.8 (Huovinen et al., 1995). The key difference was the number of annotated *dfrA* copies by RAST, two copies in the two low TMP resistant isolates and two or three copies for isolates in the medium and high resistant isolates. Additional *dfrA* alleles may be on integrons or plasmids which encode TMP resistant DHFRs which are not susceptible to TMP (Krucinska et al., 2022; Labar et al., 2012). This further allows for folate reduction to occur under TMP antibiotic stress, therefore a higher copy numbers of the *dfrA* gene may be expected in higher resistant isolates, however more isolates are needed to investigate this further (Grape et al., 2007; Huovinen et al., 1995; Li et al., 2020; Partridge et al., 2018; Sköld, 2000). To confirm number of *dfrA* genes and if they are on mobile genetic elements, RAST annotated *dfrA* genes were run through BLASTn. Table 3.6 showed that isolates contained

one or two copies of the gene. This could be further determined using quantitative polymerase chain reactions to investigate the gene response to TMP (Shi et al., 2021).

The OrthoVenn and CARD results from Table 3.4 and gene similarities in Figure 3.8 showed all TMP resistance isolates contained *dfrA* variants. This was confirmed by RAST annotations where BLASTn was used to validate annotations. RAST showed potential *dfrA* annotations of genes to be a *folA* gene. As stated above in Section 3.2.7, using multiple databases offers different levels of evidence to provide more reliable confirmation of gene identity and function (Griesemer et al., 2018). Plasmids may often carry determinants which are responsible for inducing the resistance to many antibiotics (Geurtsen et al., 2022). BLASTn located the *dfrA* genes to be on plasmids in other *E. coli* isolates. This is important to investigate since resistance genes can be transferred from strains via mobile elements (Ibrahim et al., 2023; Khanal et al., 2024; Toro et al., 2014).

Isolates of *E. coli* from human and clinical isolates investigating TMP resistance report the presence of at least one *dfrA* gene (Brolund et al., 2010; Mazurek et al., 2015; Somorin et al., 2022). The multiple *dfrA* genes shown by isolate 01 from the medium TMP resistance group does not guarantee high TMP resistance (Canal et al., 2016; Somorin et al., 2022). Multiple *dfrA* genes are seen in human UTI strains however only one may be expressed (Brolund et al., 2010). Biochemical and structural studies on TMP resistance alleles show that different *dfrA* genes can yield different resistance levels (Brolund et al., 2010; Krucinska et al., 2022; Lee et al., 2001; Somorin et al., 2022). Therefore, the differences seen between the two *dfrA* variants within each of the three TMP resistance levels may dependent on which *dfrA* variants are present in the strain and how they are expressed (Brolund et al., 2010; Krucinska et al., 2022; Lee et al., 2001). Future research may investigate how transcription analysis may be done to investigate how the expression levels of the *dfrA* genes differ from housekeeping genes in the strain (Mazurek et al., 2015). Transcriptomics using RNA-Sequencing may also be used to investigate if a *dfrA* variant is expressed more than the other, such as in isolate 01 where *dfrA5* and *dfrA15* were identified (Manoharan-Basil et al., 2025; Poonawala et al., 2024).

Large clinical studies investigating UPEC showed *dfrA1*, *dfrA12*, and *dfrA17* variants were prevalent TMP associated resistance genes (Lee et al., 2001; Li et al., 2020; Poey et al., 2024; Yu et al., 2004). Isolates 57 and 98 contained *dfrA1* and isolate 23 contained *dfrA17*. A less frequently seen variant in resistant studies is *dfrA5* (Šeputienė et al., 2010). Isolates 01, 25, and 79 contained this variant. The variant *dfrA15* is not commonly listed among *dfrA* variants associated in *E. coli* but was seen in isolate 01 (Barraud & Ploy, 2015; Grape et al., 2007; Lacotte et al., 2017). Phenotypic TMP resistance thresholds categorised by low, medium, and high

criterium may be a result of the diversity of *dfrA* variants and if they are commonly seen in *E. coli* strains (Barraud & Ploy, 2015).

Nucleotide differences in genes may show recent divergence where protein differences may show ancient evolutionary relationships from amino acid changes (Huntzinger & Izaurralde, 2011). Phylogenetic trees shown in Figure 3.9 and Figure 3.10 showed isolate *dfrA* variants were positioned irrespective of the TMP resistance level rather it was the type of *dfrA* that determined the positioning. The bootstrap values were ≥ 97% for the nucleotide maximum likelihood the protein maximum likelihood bootstrap values were 100%. These values suggest clades were strongly supported (Hillis & Bull, 1993). TMP resistance may not be solely due to the presence of *dfrA* genes, suggesting other mechanisms other than antifolate target mutations may be involved in resistance (Somorin et al., 2022).

3.4.5 Novel Resistance Determinants and dfrA Genes Involvement in TMP Resistance

The whole genome analysis from OrthoVenn3 cluster and CARD and investigations suggests TMP resistance in *E. coli* is the result of multiple mechanisms and presence of certain genes. Such as the presence of efflux pumps (as shown in Table 3.4 and Figure 3.8), which can reduce antibiotic concentrations within cells and allow for resistance (Blair et al., 2015; Li & Nikaido, 2009). The acquisition of genes such as *dfrA* variants on plasmids may provide antibiotic target replacements while integron associated *sul* genes allow for multidrug resistance (Lacotte et al., 2017) . This highlights the involvement of intrinsic chromosomal mechanisms and horizontally acquired mobile elements such as plasmids in resistance (Rasko et al., 2008; Touchon et al., 2020).

3.5 Conclusion

This chapter highlights the importance of investigating phenotypic TMP resistance with its genomic profiles. Genome annotations of the six isolates showed the presence of AMR genes, including several *dfrA* variants. Their presence did not determine the levels of TMP resistance established by phenotypic AST results presented in Chapter 2. Phylogenetic trees constructed from *dfrA* nucleotides and protein sequences showed clades determined by *dfrA* variant rather than TMP resistance level. Future studies should increase the number of isolates within each TMP resistant level and incorporate functional analysis to explore the roles of plasmids, efflux pumps and other AMR pathways. In addition, transcriptomic analysis of AMR and housekeeping genes would provide valuable information into how TMP is ineffective against UPEC strains.

Chapter 4 Final Discussion

Antimicrobial resistance (AMR) is a growing public health crisis, contributing to rising mortality and morbidity (Manna et al., 2021). Resistance is an emerging public health crisis, with increasing numbers associated with mortality and morbidity involving AMR infections. As bacteria only have several antibiotic target proteins, they are nearing complete resistance to several antibiotic molecules with few genetic mutations (Manna et al., 2021; Tamer et al., 2019). Almost all human pathogens exhibit AMR mechanisms causing a heavy burden on health care systems globally (Melnyk et al., 2015). Through genetic mutations and the acquisition of new genes, bacteria have become more resistant to several crucial first line antibiotics used to treat them (Bunduki et al., 2021; Bushby & Hitchings, 1968; Nolan et al., 2015).

Trimethoprim (TMP) was once a commonly used antibiotic to treat uropathogenic *Escherichia coli* (UPEC) infections (Flores-Mireles et al., 2015). However, high levels of resistance observed by pathogens have resulted in TMP being an ineffective antibiotic, which highlights the importance to understand AMR within this public health crisis (D. S. Lee et al., 2018b; Manna et al., 2021; Tamer et al., 2019).

Currently, there is a limited knowledge of the resistance mechanisms on New Zealand (NZ) UPEC isolates. This study identified the resistance mechanisms of UPEC isolates from Middlemore Hospital (Auckland, NZ) through minimum inhibitory concentration (MIC) testing and comparative genomic analysis. This study aimed to confirm established TMP resistance mechanisms, while exploring novel resistance mechanisms among these NZ isolates.

4.1 Antibiotic Susceptibility Testing of *E. coli*

RQ1: What antibiotics are UPEC isolates resistant to and at what concentrations?

To confirm UPEC isolates from Middlemore Hospital exhibited TMP resistance, disk diffusion antibiotic susceptibility testing (AST) was done. Of the 106 isolates tested, 98 retained TMP resistance with 82 of these isolates resistant to the combination trimethoprim-sulfamethoxazole (TMP-SMX). These results are consistent with findings from LabPLUS (2023) and Heffernan et al. (2009).

Several isolates in the present study were classified as being susceptible or intermediate to nitrofurantoin and amoxicillin-clavulanic acid with several isolates showing resistant colonies within the zone of inhibition of the meropenem disk. These resistance phenotypes may be investigated further as stated in Section 4.3 to understand implications on clinical empirical

prescriptions. Resistant colonies to meropenem may be a cause of concern for future infection management given LabPLUS (2023) reported 100% susceptibility among clinical *E. coli* isolates (Table 1.3). This suggests the potential for carbapenem resistance by NZ clinical isolates which may impact infection management in the future.

RQ2: How does the growth rate vary between TMP resistant UPEC isolates (determined by RQ1) in the presence of varying concentrations of TMP at 37°C?

To determine if each isolate exhibited phenotypic differences in TMP resistance, OD curves were generated from increasing TMP concentrations during MIC testing. Each of the 74 TMP resistant isolates from disk diffusion assay AST showed differences in growth over 24 hours and generally showed lower maximum levels of growth as the TMP concentration increased. The curves generated by OD readings suggest that several isolates are able to withstand over 100 times the clinical breakpoint as stated in CLSI (2023). These OD statistical models would need to be supported by viable counts in future research.

To determine which isolates exhibited low, medium, and high TMP, a criterion was created from the OD curves generated from MIC testing. To our knowledge, neither the extent of TMP resistance in *E. coli* exceed well above the clinical breakpoint have been investigated, especially in NZ. Results from Chapter 2 show that several of the resistant isolates were able to tolerate TMP concentrations 128 times above the clinical breakpoint. Therefore, these isolates were selected for WGS to investigate differences in isolate genomes within and between resistance levels.

4.2 Whole Genome Sequencing of *E. coli*

RQ3: What are the genetic differences of the whole genomes of differing levels of TMP resistant UPEC strains in the absence of the antibiotic?

To determine the genetic differences of the isolates whole genome analysis was carried out. The identification of potential novel resistance determinants was done using CARD and OrthoVenn3 to identify genes of interest and protein clusters across resistance levels. The presence of the *dfrA* gene was confirmed by RAST annotation and BLASTn analysis before nucleotide and protein maximum likelihood phylogenetic trees were constructed using the Bootstrap method. The expression levels of plasmids, efflux pumps and other AMR pathways may provide useful information in determining genetic influences on phenotypic TMP resistance levels.

4.3 Future Directions

Areas for the direction of further research were identified from the summary of results described above. These areas have been presented below.

- **Larger sample size.** The present study used a small sample size of 106 isolates, therefore increasing the sample size may help understand the resistance mechanisms exhibited by NZ UPEC.
    - Using more isolates during OD analysis may be used to validate trends seen by each of the three resistance levels during AUC statistical analysis.
    - Obtaining UPEC isolates from various hospitals around NZ may show regional differences in resistance phenotypes which could then be used to direct current empirical antibiotic stewardship. For example, to determine if resistance and empirical treatment is high in urban areas as explored by Ussher et al. (2020).
- **Comparisons with *E. coli* strains isolated from non-human urine samples.** Comparative genomics can be used to identify if resistance mechanisms are the same or differ depending on where isolates were obtained.
    - This may be done within clinical samples containing intestinal or extraintestinal pathogenic *E. coli* strains to investigate if strains isolated from different body sites vary in their exhibited resistance mechanisms.
    - Comparisons may be made with other strains obtained from the environment such as from soils, water, or other mammals to investigate if there are environmental influences on *E. coli* resistance mechanisms.
    - Construction of phylogenetic trees to estimate evolutionary relationships and genetic similarities between these *E. coli* strains.
- **Use of metadata.** The present study did not include metadata of the patient each isolate was derived from.
    - The use of metadata will help create a greater understanding of the effects of patient environmental and genetic factors on the development of antibiotic resistant UPEC in NZ.
    - The use of metadata in future research may also aid in clinical applications such as directing appropriate antibiotic therapies for certain ethnicities and health discrepancies.
- **Gene and protein expression.** The present study identified the presence of several resistance genes of interest using WGS and comparative genomics. However, there were no observable differences between the three levels of TMP resistance. Therefore,

the following may be used to investigate the expression levels of these genes to identify if there are differences within levels:

  - Transcriptomics has been revolutionised by the development of high-throughput next-generation sequencing by enabling the analysis of RNA through sequencing of millions of short reads from the ends of complementary DNA (cDNA) from sample RNA fragments (Kukurba & Montgomery, 2015; Li & Dewey, 2011; Wang et al., 2009). This may be used to identify if there are differences in gene expression levels at varying TMP concentrations (i.e., exceeding the clinical breakpoint of 32 µg/mL). This could determine if strains exhibiting high resistance show upregulation of certain resistance genes compared to low resistance strains. This approach could be investigated to determine whether reduced expression of housekeeping genes allows for the phenotypic level of resistance.
  - Each isolate demonstrated typical biological functions. Therefore, investigating the effect of TMP on metabolic pathways using metabolomics may be used to investigate how *E. coli* metabolism changes in response to TMP stress in their environment (Mathieu et al., 2016; Zampieri et al., 2017). This could be done by measuring the effect of isolate folic acid in the presence of TMP. Folic acid is involved in metabolic functions which are essential for cell survival (Fernández-Villa et al., 2019; Shi et al., 2021).
  - The function of plasmids presented in Table 3.2 may be interpreted to confirm the function and involvement in AMR and the expression of genes on the plasmid in response to antibiotic stress.
  - Suspected efflux pumps may be investigated for their role in TMP resistance using proteomics alongside gene knockout. These genes in resistant strains may be suppressed to investigate if resistance is lost and genes may be activated in susceptible strains to investigate if resistance is induced.
  - Investigations of structural and mechanical studies involved in DHFR enzymes to understand the effect of protein differences in *dfrA* variants and levels of TMP resistance (Krucinska et al., 2022).

- **Investigation of other folate pathway genes.** Evolutionary experiments identified point mutations in *folA* as mentioned in Section 1.3.5.2. Therefore, future directions can investigate variants of *folA* genes in relation to high levels of TMP exposure as this may play a role in TMP susceptibility (Tamer et al., 2019). Multiple sequence alignments of *folA* variants may be done using MEGA11 MUSCLE to identify point mutations which

may highlight nucleotide changes which correlate to the phenotypic level of resistance (Podnecky et al., 2017; Sierra et al., 2020).

- **Comparison with international data.** Comparative genomics may be used to understand if there is novelty of TMP resistance levels in NZ isolates from this study to other studies investigating TMP resistance in *E. coli* from different countries.
    - A curated database such as PubMLST may be used for genotypic and epidemiological analysis of the tested isolates. This could further findings established in Section 2.2.3 of this study and contribute to the understanding of global antibiotic resistance and direct national dispensing rates.
    - Utilising statistical analysis outlined in international data to interpret growth curves would allow for quantification comparison of isolates in response to the tested TMP concentrations (Angermayr et al., 2022; Chandrasekaran & Jiang, 2019).
- **Investigations involving sulfamethoxazole on *E. coli* isolates.** TMP was the primary focus of the present study. As TMP is commonly prescribed in combination with sulfamethoxazole in clinical applications, future studies may investigate the relationship between the two antifolate antibiotics as resistance requires two independent mutational events to occur for resistance to develop (Murray et al., 1982).
    - Identify if TMP-SMX resistant strains demonstrate different phenotypic resistance OD curves in the presence of one or both antibiotics.
    - Investigating if SMX resistant strains exhibit the same or different resistance mechanisms to TMP resistant strains during whole genome analysis. This could be further investigated using gene expression tools such as transcriptomics as mentioned above.
- **Wider clinical applications.**
    - If used, empirical treatments should be updated to account for resistance rates for antibiotics used before sample culturing and sensitivity results are available.
    - Encouraging practitioners or clinic communication with molecular pathologists. By doing this, practitioners who prescribe antibiotics to patients are informed of resistance rates and updated with criteria that can be used for empirical treatment and/or culture and sensitivity testing. This may allow for appropriate patient treatment and efficiency during laboratory testing with high influx samples.
    - Educating the public on AMR such as the annual LabPlus susceptibility results, therefore informing patients on treatment options, such as if empirical treatment is suitable prior to culture and susceptibility testing.

4.4 Conclusions

To conclude, the present study is the first to utilise levels of resistance and comparative genomics to study antibiotic resistant *Escherichia coli* isolates from urinary tract infections in New Zealand. Findings have suggested that there are varying levels of resistance above the clinical breakpoint. Whole genome analysis of resistant isolates and comparative genomics showed isolates contained at least one *dfrA* variant, which is an established contributor to trimethoprim resistance in *E. coli*. Furthermore, efflux pumps may contribute to their level of resistance and investigating their gene expression may be of interest in future research.

Overall, this initial study of trimethoprim resistant strains of uropathogenic *E. coli* from New Zealand has provided a snapshot of the genomic characteristics of these strains. There are many areas of future directions which may be applied to fully understand uropathogenic *E. coli* and its mechanisms of antibiotic resistance.

References

Abdelwahed, E. K., Hussein, N. A., Moustafa, A., Moneib, N. A., & Aziz, R. K. (2022). Gene networks and pathways involved in Escherichia coli response to multiple stressors. *Microorganisms, 10*(9), 1-23. https://doi.org/10.3390/microorganisms10091793

Adrian, P. V., & Klugman, K. P. (1997). Mutations in the dihydrofolate reductase gene of trimethoprim-resistant isolates of Streptococcus pneumoniae. *Antimicrobial Agents and Chemotherapy, 41*(11), 2406-2413. https://doi.org/10.1128/aac.41.11.2406

Ahmed, S. K., Hussein, S., Qurbani, K., Ibrahim, R. H., Fareeq, A., Mahmood, K. A., & Mohamed, M. G. (2024). Antimicrobial resistance: Impacts, challenges, and future prospects. *Journal of Medicine, Surgery, and Public Health, 2*, 100081. https://doi.org/10.1016/j.glmedi.2024.100081

Al Lawati, H., Blair, B. M., & Larnard, J. (2024). Urinary Tract Infections: Core Curriculum 2024. *American Journal of Kidney Diseases, 83*(1), 90-100. https://doi.org/10.1053/j.ajkd.2023.08.009

Alcock, B. P., Huynh, W., Chalil, R., Smith, K. W., Raphenya, Amogelang R., Wlodarski, M. A., Edalatmand, A., Petkau, A., Syed, S. A., Tsang, K. K., Baker, S. J. C., Dave, M., McCarthy, Madeline C., Mukiri, K. M., Nasir, J. A., Golbon, B., Imtiaz, H., Jiang, X., Kaur, K., Kwong, M., Liang, Z. C., Niu, K. C., Shan, P., Yang, J. Y. J., Gray, Kristen L., Hoad, G. R., Jia, B., Bhando, T., Carfrae, Lindsey A., Farha, Maya A., French, S., Gordzevich, R., Rachwalski, K., Tu, Megan M., Bordeleau, E., Dooley, D., Griffiths, E., Zubyk, H. L., Brown, E. D., Maguire, F., Beiko, Robert G., Hsiao, W. W. L., Brinkman, F. S. L., Van Domselaar, G., & McArthur, A. G. (2022). CARD 2023: expanded curation, support for machine learning, and resistome prediction at the Comprehensive Antibiotic Resistance Database. *Nucleic Acids Research, 51*(1), 690-699. https://doi.org/10.1093/nar/gkac920

Alekshun, M. N., & Levy, S. B. (2007). Molecular mechanisms of antibacterial multidrug resistance. *Cell 128*(6), 1037-1050. https://doi.org/10.1016/j.cell.2007.03.004

Altenhoff, A. M., & Dessimoz, C. (2009). Phylogenetic and Functional Assessment of Orthologs Inference Projects and Methods. *PLOS Computational Biology, 5*(1), e1000262. https://doi.org/10.1371/journal.pcbi.1000262

Ambrose, S. J., & Hall, R. M. (2021a). dfrA trimethoprim resistance genes found in gram-negative bacteria: compilation and unambiguous numbering. *Journal of Antimicrobial Chemotherapy, 76*(11), 2748-2756. https://doi.org/10.1093/jac/dkab212

Ambrose, S. J., & Hall, R. M. (2021b). Origin of the dfrA44 trimethoprim resistance gene. *The Journal of Antimicrobial Chemotherapy, 76*(12), 3312-3314. https://doi.org/10.1093/jac/dkab330

Andrews, J. M. (2001). Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy, 48*(suppl_1), 5-16. https://doi.org/10.1093/jac/48.suppl_1.5

Angermayr, S. A., Pang, T. Y., Chevereau, G., Mitosch, K., Lercher, M. J., & Bollenbach, T. (2022). Growth-mediated negative feedback shapes quantitative antibiotic response. *Molecular Systems Biology, 18*(9), e10490. https://doi.org/10.15252/msb.202110490

Arafi, V., Hasani, A., Sadeghi, J., Varshochi, M., Poortahmasebi, V., Hasani, A., & Hasani, R. (2023). Uropathogenic Escherichia coli endeavors: an insight into the characteristic features, resistance mechanism, and treatment choice. *Archives of Microbiology 205*(6), 226. https://doi.org/10.1007/s00203-023-03553-5

Arden, K., Rosanowski, S. M., Laven, R. A., & Mueller, K. R. (2024). Dairy farmer, engagement and understanding of One Health and antimicrobial resistance - a pilot survey from the lower north island of Aotearoa New Zealand. *One Health Outlook, 6*(1), 1-13. https://doi.org/10.1186/s42522-024-00107-7

Arslan, M., Tezcan, E., Camci, H., & Avci, K. (2021). Effect of DNA Concentration on Band Intensity and Resolution in Agarose Gel Electrophoresis. *Van Sağlık Bilimleri Dergisi, 13*, 326-333. https://doi.org/10.52976/vansaglik.969547

Ashiru-Oredope, D., & Hopkins, S. Antimicrobial resistance: Moving from professional engagement to public action. *70*(11), 2927-2930. https://doi.org/10.1093/jac/dkv297

Axelsson, C., Ann-Sofi, R.-H., & and Nilson, B. (2020). Rapid detection of antibiotic resistance in positive blood cultures by MALDI-TOF MS and an automated and optimized MBT-ASTRA protocol for Escherichia coli and Klebsiella pneumoniae. *Infectious Diseases, 52*(1), 45-53. https://doi.org/10.1080/23744235.2019.1682658

Axelsson, C., Nilson, B., & Rehnstam-Holm, A.-S. (2024). Efficient Absorbance-Based Assay for Rapid Antibiotic Susceptibility Testing of Enterobacterales. *Antibiotics, 13*(9), 852. https://doi.org/10.3390/antibiotics13090852

Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., Formsma, K., Gerdes, S., Glass, E. M., Kubal, M., Meyer, F., Olsen, G. J., Olson, R., Osterman, A. L., Overbeek, R. A., McNeil, L. K., Paarmann, D., Paczian, T., Parrello, B., Pusch, G. D., Reich, C., Stevens, R., Vassieva, O., Vonstein, V., Wilke, A., & Zagnitko, O. (2008). The RAST Server: Rapid Annotations using Subsystems Technology. *BMC Genomics, 9*(1), 75. https://doi.org/10.1186/1471-2164-9-75

Bafna, P., Deepanjali, S., Mandal, J., Balamurugan, N., Swaminathan, R. P., & Kadhiravan, T. (2020). Reevaluating the true diagnostic accuracy of dipstick tests to diagnose urinary tract infection using Bayesian latent class analysis. *PLoS ONE, 15*(12), 1-12. https://doi.org/10.1371/journal.pone.0244870

Baharoglu, Z., & Mazel, D. (2014). SOS, the formidable strategy of bacteria against aggressions. *FEMS Microbiology Reviews, 38*(6), 1126-1145. https://doi.org/10.1111/1574-6976.12077

Baker, C. N., Stocker, S. A., Culver, D. H., & Thornsberry, C. (1991). Comparison of the E Test to agar dilution, broth microdilution, and agar diffusion susceptibility testing techniques by using a special challenge set of bacteria. *J Clin Microbiol, 29*(3), 533-538. https://doi.org/10.1128/jcm.29.3.533-538.1991

Balouiri, M., Sadiki, M., & Ibnsouda, S. K. (2016). Methods for in vitro evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis, 6*(2), 71-79. https://doi.org/10.1016/j.jpha.2015.11.005

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., Lesin, V. M., Nikolenko, S. I., Pham, S., Prjibelski, A. D., Pyshkin, A. V., Sirotkin, A. V., Vyahhi, N., Tesler, G., Alekseyev, M. A., & Pevzner, P. A. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol, 19*(5), 455-477. https://doi.org/10.1089/cmb.2012.0021

Baquero, F. (2001). Low-level antibacterial resistance: a gateway to clinical resistance. *Drug Resistance Updates, 4*(2), 93-105. https://doi.org/10.1054/drup.2001.0196

Baquero, F., & Blázquez, J. (1997). Evolution of antibiotic resistance. *Trends in Ecology & Evolution, 12*(12), 482-487. https://doi.org/10.1016/S0169-5347(97)01223-8

Baquero, F., Martínez, J. L., Lanza, V. F., Rodríguez-Beltrán, J., Galán, J. C., Millán, A. S., Cantón, R., & Coque, T. M. (2021). Evolutionary Pathways and Trajectories in Antibiotic Resistance. *Clinical Microbiology Reviews, 34*(4), e00050-00019. https://doi.org/10.1128/CMR.00050-19

Barraud, O., & Ploy, M.-C. (2015). Diversity of Class 1 Integron Gene Cassette Rearrangements Selected under Antibiotic Pressure. *Journal of Bacteriology, 197*(13), 2171-2178. https://doi.org/doi:10.1128/jb.02455-14

Bertranda, R. L. (2019). Lag phase is a dynamic, organized, adaptive, and evolvable period that prepares bacteria for cell division. *Journal of Bacteriology, 201*(7), 1-21. https://doi.org/10.1128/JB.00697-18

Bielecki, P., Muthukumarasamy, U., Eckweiler, D., Bielecka, A., Pohl, S., Schanz, A., Niemeyer, U., Oumeraci, T., Neuhoff, N. v., Ghigo, J. M., & Häussler, S. (2014). In vivo mRNA profiling of uropathogenic Escherichia coli from diverse phylogroups reveals common and group-specific gene expression profiles. *mBio, 5*(4), 1-12. https://doi.org/10.1128/mBio.01075-14

Bilsen, M. P., Jongeneel, R. M. H., Schneeberger, C., Platteel, T. N., van Nieuwkoop, C., Mody, L., Caterino, J. M., Geerlings, S. E., Koves, B., Wagenlehner, F., Conroy, S. P., Visser, L. G., & Lambregts, M. M. C. (2023). Definitions of urinary tract infection in current research: A systematic review. *Open Forum Infectious Diseases 10*(7), 1-8. https://doi.org/10.1093/ofid/ofad332

Birkegård, A. C., Halasa, T., Toft, N., Folkesson, A., & Græsbøll, K. (2018). Send more data: a systematic review of mathematical models of antimicrobial resistance. *Antimicrobial Resistance & Infection Control, 7*(1), 117. https://doi.org/10.1186/s13756-018-0406-1

Blair, J. M. A., Webber, M. A., Baylay, A. J., Ogbolu, D. O., & Piddock, L. J. V. (2015). Molecular mechanisms of antibiotic resistance. *Nature Reviews Microbiology, 13*(1), 42-51. https://doi.org/10.1038/nrmicro3380

Bogart, L. M., Berry, S. H., & Clemens, J. Q. (2007). Symptoms of interstitial cystitis, painful bladder syndrome and similar diseases in women: A systematic review. *Journal of Urology, 177*(2), 450-456. https://doi.org/10.1016/j.juro.2006.09.032

Boolchandani, M., D'Souza, A. W., & Dantas, G. (2019). Sequencing-based methods and resources to study antimicrobial resistance. *Nature Reviews Genetics 20*(6), 356-370. https://doi.org/10.1038/s41576-019-0108-4

BpacNZ. (2017). *Antibiotics: Choices for Common Infections*. Best Practice Inc. [Accessed on 04 May 2023] from https://bpac.org.nz/antibiotics/guide.aspx

BpacNZ. (2021). *The diagnosis and management of symptomatic lower UTIs in adults*. [Accessed 06 October 2023] from https://bpac.org.nz/2021/uti.aspx

Braz, V. S., Melchior, K., & Moreira, C. G. (2020). Escherichia coli as a multifaceted pathogenic and versatile bacterium. *Frontiers in Cellular & Infection Microbiology, 10*, 1-9. https://doi.org/10.3389/fcimb.2020.548492

Bretonnière, C., Maitte, A., Caillon, J., Potel, G., Boutoille, D., Jacqueline, C., & Guitton, C. (2016). MIC score, a new tool to compare bacterial susceptibility to antibiotics application to the comparison of susceptibility to different penems of clinical strains of Pseudomonas aeruginosa. *The Journal of Antibiotics, 69*(11), 806-810. https://doi.org/10.1038/ja.2016.38

Brolund, A., Grape, M., Sundqvist, M., & Kahlmeter, G. (2010). Molecular characterisation of trimethoprim resistance in Escherichia coli and Klebsiella pneumoniae during a two year intervention on trimethoprim use. *PLoS ONE, 5*(2), 1-5. https://doi.org/10.1371/journal.pone.0009233

Brosnan, Margaret E., MacMillan, L., Stevens, Jennifer R., & Brosnan, John T. (2015). Division of labour: how does folate metabolism partition between one-carbon metabolism and amino acid oxidation? *Biochemical Journal, 472*(2), 135-146. https://doi.org/10.1042/bj20150837

Buckner, M. M. C., Ciusa, M. L., & Piddock, L. J. V. (2018). Strategies to combat antimicrobial resistance: anti-plasmid and plasmid curing. *FEMS Microbiology Reviews, 42*(6), 781-804. https://doi.org/10.1093/femsre/fuy031

Bunduki, G. K., Heinz, E., Phiri, V. S., Noah, P., Feasey, N., & Musaya, J. (2021). Virulence factors and antimicrobial resistance of uropathogenic Escherichia coli (UPEC) isolated from urinary tract infections: a systematic review and meta-analysis. *BMC Infectious Diseases, 21*(1), 753. https://doi.org/10.1186/s12879-021-06435-7

Bush, K., & Bradford, P. A. (2016). β-Lactams and β-Lactamase Inhibitors: An Overview. *Cold Spring Harb Perspect Med, 6*(8), 1-22. https://doi.org/10.1101/cshperspect.a025247

Bushby, S. R., & Hitchings, G. H. (1968). Trimethoprim, a sulphonamide potentiator. *Br J Pharmacol Chemother, 33*(1), 72-90. https://doi.org/10.1111/j.1476-5381.1968.tb00475.x

Canal, N., Meneghetti, K. L., de Almeida, C. P., da Rosa Bastos, M., Otton, L. M., & Corção, G. (2016). Characterization of the variable region in the class 1 integron of antimicrobial-resistant Escherichia coli isolated from surface water. *Brazilian Journal of Microbiology, 47*(2), 337-344. https://doi.org/10.1016/j.bjm.2016.01.015

Carmona-Cartaya, Y., Hidalgo-Benito, M., Borges-Mateus, L. M., Pereda-Novales, N., González-Molina, M. K., & Quiñones-Pérez, D. (2022). Community-Acquired Uropathogenic Escherichia coli, Antimicrobial Susceptibility, and Extended-Spectrum Beta-Lactamase Detection. *MEDICC Review, 24*(2), 20-25. https://doi.org/10.37757/mr2022.v24.n2.2

Carriço, J. A., Sabat, A. J., Friedrich, A. W., & Ramirez, M. (2013). Bioinformatics in bacterial molecular epidemiology and public health: databases, tools and the next-generation sequencing revolution. *Euro Surveillance, 18*(4), 20382. https://doi.org/10.2807/ese.18.04.20382-en

Catalán, P., Wood, E., Blair, J. M. A., Gudelj, I., Iredell, J. R., & Beardmore, R. E. (2022). Seeking patterns of antibiotic resistance in ATLAS, an open, raw MIC database with patient metadata. *Nature Communications, 13*(1), 2917. https://doi.org/10.1038/s41467-022-30635-7

Cellier-Goetghebeur, S., Lafontaine, K., Lemay-St-Denis, C., Tsamo, P., Bonneau-Burke, A., Copp, J. N., & Pelletier, J. N. (2022). Discovery of highly trimethoprim-resistant DfrB dihydrofolate reductases in diverse environmental settings suggests an evolutionary advantage unrelated to antibiotic resistance. *Antibiotics, 11*(12), 1-12. https://doi.org/10.3390/antibiotics11121768

Chagneau, C. V., Massip, C., Bossuet-Greif, N., Fremez, C., Motta, J.-P., Shima, A., Besson, C., Le Faouder, P., Cénac, N., Roth, M.-P., Coppin, H., Fontanié, M., Martin, P., Nougayrède, J.-P., & Oswald, E. (2021). Uropathogenic E. coli induces DNA damage in the bladder. *PLoS Pathogens, 17*(2), 1-18. https://doi.org/10.1371/journal.ppat.1009310

Chandrasekaran, S., & Jiang, S. C. (2019). A dose response model for quantifying the infection risk of antibiotic-resistant bacteria. *Sci Rep, 9*(1), 17093. https://doi.org/10.1038/s41598-019-52947-3

Chen, A. K. D., Duffy, E. J., Ritchie, S. R., & Thomas, M. G. (2019). Diagnostic accuracy and adherence to treatment guidelines in adult inpatients with urinary tract infections in a tertiary hospital. *Journal of Pharmacy Practice & Research, 49*(3), 246-253. https://doi.org/10.1002/jppr.1508

Chen, Y. C., Chang, C. C., Chiu, T. H. T., Lin, M. N., & Lin, C. L. (2020). The risk of urinary tract infection in vegetarians and non-vegetarians: a prospective study. *Scientific Reports, 10*(1). https://doi.org/10.1038/s41598-020-58006-6

Choi, U., & Lee, C.-R. (2019). Distinct Roles of Outer Membrane Porins in Antibiotic Resistance and Membrane Integrity in Escherichia coli. *Frontiers in Microbiology, 10*. https://doi.org/10.3389/fmicb.2019.00953

Christaki, E., Marcou, M., & Tofarides, A. (2020). Antimicrobial resistance in bacteria: Mechanisms, evolution, and persistence. *Journal of Molecular Evolution, 88*(1), 26-40. https://doi.org/10.1007/s00239-019-09914-3

Churchill, K. (2023). The management of recurrent urinary tract infections within a nurse-led urology team. *British Journal of Nursing, 32*(9), 6-12. https://doi.org/10.12968/bjon.2023.32.9.S6

CLSI. (2023). *Performance Standards for Antimicrobial Susceptibility Testing* (33 ed.). Clinical and Laboratory Standards Institute. [Accessed on 04 May 2023] from http://em100.edaptivedocs.net/GetDoc.aspx?doc=CLSI%20M100%20ED33:2023&scope=user

Coll, F., Gouliouris, T., Blane, B., Yeats, C. A., Raven, K. E., Ludden, C., Khokhar, F. A., Wilson, H. J., Roberts, L. W., Harrison, E. M., Horner, C. S., Le, T. H., Nguyen, T. H., Nguyen, V. T., Brown, N. M., Holmes, M. A., Parkhill, J., Estee Török, M., & Peacock, S. J. (2024). Antibiotic resistance determination using Enterococcus faecium whole-genome sequences: a diagnostic accuracy study using genotypic and phenotypic data. *The Lancet Microbe*. https://doi.org/10.1016/S2666-5247(23)00297-5

Cookson, A. L., Rogers, L. E., Collis, R. M., Brightwell, G., Marshall, J. C., Biggs, P. J., Wilkinson, D. A., Devane, M., Stott, R., & Kamke, J. (2022). Whole-genome sequencing and virulome analysis of Escherichia coli isolated from New Zealand environments of contrasting observed land use. *Applied and Environmental Microbiology, 88*(9), 1-19. https://doi.org/10.1128/aem.00277-22

Critchley, I. A., Cotroneo, N., Pucci, M. J., & Mendes, R. (2019). The burden of antimicrobial resistance among urinary tract isolates of Escherichia coli in the United States in 2017. *PLoS ONE, 14*(12), e0220265. https://doi.org/10.1371/journal.pone.0220265

Croxen, M. A., & Finlay, B. B. (2010). Molecular mechanisms of Escherichia coli pathogenicity. *Nature Reviews Microbiology, 8*(1), 26-38. https://doi.org/10.1038/nrmicro2265

Crum, E., Merchant, Z., Ene, A., Miller-Ensminger, T., Johnson, G., Wolfe, A. J., & Putonti, C. (2023). Coliphages of the human urinary microbiota. *PLoS ONE, 17*(4), 1-16. https://doi.org/10.1371/journal.pone.0283930

Dadzie, I., Abiade, V., Takyi-Amuah, E., Adusei, R., Quansah, E., & Puopelle Dakorah, M. (2019). The effectiveness of dipstick for the detection of urinary tract infection. *Canadian Journal of Infectious Diseases and Medical Microbiology, 2019*, 1-6. https://doi.org/10.1155/2019/8642628

Darby, E. M., Trampari, E., Siasat, P., Gaya, M. S., Alav, I., Webber, M. A., & Blair, J. M. A. (2022). Molecular mechanisms of antibiotic resistance revisited. *Nature Reviews Microbiology*. https://doi.org/10.1038/s41579-022-00820-y

Delhaye, A., Collet, J.-F., & Laloux, G. (2019). A Fly on the Wall: How Stress Response Systems Can Sense and Respond to Damage to Peptidoglycan. *Frontiers in Cellular and Infection Microbiology, 9*, 1-13. https://doi.org/10.3389/fcimb.2019.00380

Deter, H. S., Hossain, T., & Butzin, N. C. (2021). Antibiotic tolerance is associated with a broad and complex transcriptional response in E. coli. *Scientific Reports, 11*(1), 6112. https://doi.org/10.1038/s41598-021-85509-7

Diaz-Diaz, S., Recacha, E., García-Duque, A., Docobo-Pérez, F., Blázquez, J., Pascual, A., & Rodríguez-Martínez, J. M. (2022). Effect of RecA inactivation and detoxification systems on the evolution of ciprofloxacin resistance in Escherichia coli. *Journal of Antimicrobial Chemotherapy (JAC), 77*(3), 641-645. https://doi.org/10.1093/jac/dkab445

Dobrindt, U., Chowdary, M. G., Krumbholz, G., & Hacker, J. (2010). Genome dynamics and its impact on evolution of Escherichia coli. *Medical Microbiology & Immunology, 199*(3), 145-154. https://doi.org/10.1007/s00430-010-0161-2

Duffy, E., Ritchie, S., Metcalfe, S., Van Bakel, B., & Thomas, M. G. (2018). Antibacterials dispensed in the community comprise 85%-95% of total human antibacterial consumption. *Journal of Clinical Pharmacy & Therapeutics, 43*(1), 59-64. https://doi.org/10.1111/jcpt.12610

El-Halfawy, O. M., & Valvano, M. A. (2012). Non-genetic mechanisms communicating antibiotic resistance: rethinking strategies for antimicrobial drug design. *Expert Opinion on Drug Discovery, 7*(10), 923-933. https://doi.org/10.1517/17460441.2012.712512

Elbing, K. L., & Brent, R. (2019). Recipes and Tools for Culture of Escherichia coli. *Current Protocols in Molecular Biology, 125*(1), 1-19. https://doi.org/10.1002/cpmb.83

Eliopoulos, G. M., & Huovinen, P. (2001). Resistance to Trimethoprim-Sulfamethoxazole. *Clinical Infectious Diseases, 32*(11), 1608-1614. https://doi.org/10.1086/320532

Ellington, M. J., Ekelund, O., Aarestrup, F. M., Canton, R., Doumith, M., Giske, C., Grundman, H., Hasman, H., Holden, M. T. G., Hopkins, K. L., Iredell, J., Kahlmeter, G., Köser, C. U., MacGowan, A., Mevius, D., Mulvey, M., Naas, T., Peto, T., Rolain, J. M., Samuelsen, Ø., & Woodford, N. (2017). The role of whole genome sequencing in antimicrobial susceptibility testing of bacteria: report from the EUCAST Subcommittee. *Clinical Microbiology and Infection, 23*(1), 2-22. https://doi.org/10.1016/j.cmi.2016.11.012

Etefia, U. E., & Solomon, A. B. (2020). Virulence markers, phylogenetic evolution, and molecular techniques of uropathogenic Escherichia coli. *Journal of Nature and Science of Medicine, 3*(1), 13-22. https://doi.org/10.4103/JNSM.JNSM_31_19

EUCAST. (2021). *EUCAST Disk Diffusion Method*. https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/2021_manuals/Manual_v_9.0_EUCAST_Disk_Test_2021.pdf

EUCAST. (2022). *Breakpoint tables for interpretation of MICs and zone diameters*. [Accessed on 15 March 2022] from https://www.eucast.org/clinical_breakpoints

EUCAST. (2023). *Clinical breakpoints and dosing of antibiotics*. The European Committee on Antimicrobial Susceptibility Testing. [Accessed on 17 June 2023] from https://www.eucast.org/clinical_breakpoints

EUCAST Definitive Document. (1998). Methods for the determination of susceptibility of bacteria to antimicrobial agents. Terminology. *Clinical Microbiology and Infection, 4*(5), 291-296. https://doi.org/https://doi.org/10.1111/j.1469-0691.1998.tb00061.x

Fairweather, J., Findlay, M., & Isles, C. (2020). *Urinary Tract Infection. Clinical companion in nephrology* (Second ed.). Springer. [Accessed on 11 July 2023] from https://doi.org/10.1007/978-3-030-38320-6_31

Fajfr, M., Balik, M., Cermakova, E., & Bostik, P. (2020, Aug 13). Effective treatment for uncomplicated urinary tract infections with oral fosfomycin, single center four year retrospective study. *Antibiotics (Basel), 9*(8), 1-9. https://doi.org/10.3390/antibiotics9080511

Fernández-Villa, D., Aguilar, M. R., & Rojo, L. (2019). Folic acid antagonists: Antimicrobial and immunomodulating mechanisms and applications. *International Journal of Molecular Sciences, 20*(20), 1-30. https://doi.org/10.3390/ijms20204996

Fisher, C. L., Dillon, R., Anguita, E., Morris-Rosendahl, D. J., & Awan, A. R. (2022). A Novel Bead-Capture Nanopore Sequencing Method for Large Structural Rearrangement Detection in Cancer. *The Journal of Molecular Diagnostics, 24*(12), 1264-1278. https://doi.org/10.1016/j.jmoldx.2022.09.006

Flensburg, J., & Sköld, O. (1987). Massive overproduction of dihydrofolate reductase in bacteria as a response to the use of trimethoprim. *European Journal of Biochemistry, 162*(3), 473-476. https://doi.org/10.1111/j.1432-1033.1987.tb10664.x

Fling, M. E., & Richards, C. (1983). The nucleotide sequence of the trimethoprim-resistant dihydrofolate reductase gene harbored by Tn7. *Nucleic Acids Res, 11*(15), 5147-5158. https://doi.org/10.1093/nar/11.15.5147

Flores-Mireles, A. L., Walker, J. N., Caparon, M., & Hultgren, S. J. (2015). Urinary tract infections: epidemiology, mechanisms of infection and treatment options. *Nature Reviews Microbiology, 13*(5), 269-284. https://doi.org/10.1038/nrmicro3432

Foxman, B. (2014). Urinary Tract Infection Syndromes: Occurrence, Recurrence, Bacteriology, Risk Factors, and Disease Burden. *Infectious Disease Clinics of North America 28*(1), 1-13. https://doi.org/10.1016/j.idc.2013.09.003

Frisbie, L., Weissman, S. J., Kapoor, H., D'Angeli, M., Salm, A., Radcliff, J., & Rabinowitz, P. (2022). Outpatient Antibiotic Resistance Patterns of Escherichia coli Urinary Isolates Differ by Specialty Type. *Microbiology Spectrum, 10*(4), e02373-02321. https://doi.org/10.1128/spectrum.02373-21

Gabaldón, T., & Koonin, E. V. (2013). Functional and evolutionary implications of gene orthology. *Nature Reviews Genetics 14*(5), 360-366. https://doi.org/10.1038/nrg3456

Gajic, I., Kabic, J., Kekic, D., Jovicevic, M., Milenkovic, M., Culafic, D. M., Trudic, A., Ranin, L., & Opavski, N. (2022). Antimicrobial susceptibility testing: A comprehensive review of currently used methods. *Antibiotics, 11*(427), 1-26. https://doi.org/10.3390/antibiotics11040427

Galgano, M., Pellegrini, F., Catalano, E., Capozzi, L., Del Sambro, L., Sposato, A., Lucente, M. S., Vasinioti, V. I., Catella, C., Odigie, A. E., Tempesta, M., Pratelli, A., & Capozza, P. (2025). Acquired Bacterial Resistance to Antibiotics and Resistance Genes: From Past to Future. *Antibiotics, 14*(3), 1-34. https://www.mdpi.com/2079-6382/14/3/222

García, V., Lestón, L., Parga, A., García-Meniño, I., Fernández, J., Otero, A., Olsen, J. E., Herrero-Fresno, A., & Mora, A. (2023). Genomics, biofilm formation and infection of bladder epithelial cells in potentially uropathogenic Escherichia coli (UPEC) from animal sources and human urinary tract infections (UTIs) further support food-borne transmission. *One Health, 16*, 1-11. https://doi.org/10.1016/j.onehlt.2023.100558

Gati, N. S., Middendorf-Bauchart, B., Bletz, S., Dobrindt, U., & Mellmann, A. (2019). Origin and evolution of hybrid shiga toxin-producing and uropathogenic Escherichia coli strains of sequence type 141. *Journal of Clinical Microbiology, 58*(1), 1-16. https://doi.org/10.1128/jcm.01309-19

Geurtsen, J., de Been, M., Weerdenburg, E., Zomer, A., McNally, A., & Poolman, J. (2022). Genomics and pathotypes of the many faces of Escherichia coli. *FEMS Microbiology Reviews, 46*(6). https://doi.org/10.1093/femsre/fuac031

Ghosh, A., Bandyopadhyay, D., Koley, S., & Mukherjee, M. (2021). Uropathogenic Escherichia coli in India-an overview on recent research advancements and trends. *Applied Biochemistry & Biotechnology, 193*(7), 2267-2296. https://doi.org/10.1038/s41598-020-69501-1

Giordano, F., Aigrain, L., Quail, M. A., Bonfield, J. K., Davies, R. M., Jackson, D. K., Keane, T. M., Durbin, R., Ning, Z., Coupland, P., Tischler, G., Li, J., Yue, J. X., & Liti, G. (2017). De novo yeast genome assemblies from MinION, PacBio and MiSeq platforms. *Scientific Reports, 7*(1). https://doi.org/10.1038/s41598-017-03996-z

Goldstone, R. J., & Smith, D. G. E. (2017). A population genomics approach to exploiting the accessory 'resistome' of Escherichia coli. *Microbial Genomics, 3*(4), 1-15. https://doi.org/10.1099/mgen.0.000108

González, M. J., Zunino, P., Scavone, P., & Robino, L. (2020). Selection of effective antibiotics for uropathogenic Escherichia coli intracellular bacteria reduction. *Frontiers in Cellular & Infection Microbiology, 10*, 1-9. https://doi.org/10.3389/fcimb.2020.542755

Grape, M., Motakefi, A., Pavuluri, S., & Kahlmeter, G. (2007). Standard and real-time multiplex PCR methods for detection of trimethoprim resistance dfr genes in large collections of bacteria. *Clinical Microbiology and Infection, 13*(11), 1112-1118. https://doi.org/10.1111/j.1469-0691.2007.01807.x

Grazian, C. (2023). Clustering minimal inhibitory concentration data through Bayesian mixture models: An application to detect Mycobacterium tuberculosis resistance mutations. *32*(12), 2423-2439. https://doi.org/10.1177/09622802231211010

Grey, D., Hamilton-Miller, J. M., & Brumfitt, W. (1979). Incidence and mechanisms of resistance to trimethoprim in clinically isolated gram-negative bacteria. *Chemotherapy, 25*(3), 147-156. https://doi.org/10.1159/000237834

Griesemer, M., Kimbrel, J. A., Zhou, C. E., Navid, A., & D'haeseleer, P. (2018). Combining multiple functional annotation tools increases coverage of metabolic annotation. *BMC Genomics, 19*(1), 1-11. https://doi.org/10.1186/s12864-018-5221-9

Gupta, K., Hooton, T. M., Naber, K. G., Wullt, B., Colgan, R., Miller, L. G., Moran, G. J., Nicolle, L. E., Raz, R., Schaeffer, A. J., & Soper, D. E. (2011). International Clinical Practice Guidelines for the Treatment of Acute Uncomplicated Cystitis and Pyelonephritis in Women: A 2010 Update by the Infectious Diseases Society of America and the European Society for Microbiology and Infectious Diseases. *Clinical Infectious Diseases, 52*(5), e103-e120. https://doi.org/10.1093/cid/ciq257

Habib, G., Hoen, B., Tornos, P., Thuny, F., Prendergast, B., Vilacosta, I., Moreillon, P., de Jesus Antunes, M., Thilen, U., Lekakis, J., Lengyel, M., Müller, L., Naber, C. K., Nihoyannopoulos, P., Moritz, A., & Zamorano, J. L. (2009). Guidelines on the prevention, diagnosis, and treatment of infective endocarditis (new version 2009): the Task Force on the Prevention, Diagnosis, and Treatment of Infective Endocarditis of the European Society of Cardiology (ESC). Endorsed by the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) and the International Society of Chemotherapy (ISC) for Infection and Cancer. *European Heart Journal, 30*(19), 2369-2413. https://doi.org/10.1093/eurheartj/ehp285

Hamilton-Miller, J. M. T. (1979). Mechanisms and distribution of bacterial resistance to diaminopyrimidines and sulphonamides. *Journal of Antimicrobial Chemotherapy, 5*, 61-73. https://doi.org/10.1093/jac/5.Supplement_B.61

Harris, A. D., & Perencevich, E. N. (2007). A systematic review of the methods used to assess the association between appropriate antibiotic therapy and mortality in bacteremic patients. *Clinical Infectious Diseases, 45*(3), 329-337. https://doi.org/10.1086/519283

Hattab, S., Ma, A. H., Tariq, Z., Vega Prado, I., Drobish, I., Lee, R., & Yee, R. (2024). Rapid Phenotypic and Genotypic Antimicrobial Susceptibility Testing Approaches for Use in the Clinical Laboratory. *Antibiotics, 13*(8), 1-26. https://www.mdpi.com/2079-6382/13/8/786

He Ako Hiringa. (n.d). *Antibiotics- action required now to preserve effectiveness*. [Accessed on 11 April 2025] from https://epic.akohiringa.co.nz/antibiotics#section-637559a6be06d9f9aa0434fe

Heffernan, H. M., Woodhouse, R. E., Pope, C. E., & Blackmore, T. K. (2009). Prevalence and types of extended-spectrum β-lactamases among urinary Escherichia coli and Klebsiella spp. in New Zealand. *International Journal of Antimicrobial Agents, 34*(6), 544-549. https://doi.org/10.1016/j.ijantimicag.2009.07.014

Heikkilä, E., Skurnik, M., Sundström, L., & Huovinen, P. (1993). A novel dihydrofolate reductase cassette inserted in an integron borne on a Tn21-like element. *Antimicrobial Agents and Chemotherapy, 37*(6), 1297-1304. https://doi.org/10.1128/aac.37.6.1297

Helling, R. B., Goodman, H. M., & Boyer, H. W. (1974). Analysis of endonuclease R-EcoRI fragments of DNA from lambdoid bacteriophages and other viruses by agarose-gel electrophoresis. *J Virol, 14*(5), 1235-1244. https://doi.org/10.1128/jvi.14.5.1235-1244.1974

Hillis, D. M., & Bull, J. J. (1993). An Empirical Test of Bootstrapping as a Method for Assessing Confidence in Phylogenetic Analysis. *Systematic Biology, 42*(2), 182-192. https://doi.org/10.1093/sysbio/42.2.182

Hilt, E., E., & Ferrieri, P. (2022). Next generation and other sequencing technologies in diagnostic microbiology and infectious diseases. *Genes, 13*(1566), 1-24. https://doi.org/10.3390/genes13091566

Hobbs, M. R., Grant, C. C., Ritchie, S. R., Chelimo, C., Morton, S. M. B., Berry, S., & Thomas, M. G. (2017). Antibiotic consumption by New Zealand children: Exposure is near universal by the age of 5 years. *Journal of Antimicrobial Chemotherapy 72*(6), 1832-1840. https://doi.org/10.1093/jac/dkx060

Hong, J. Y., Suh, S.-W., & Shin, J. (2020). Clinical significance of urinary obstruction in critically ill patients with urinary tract infections. *Medicine, 99*(1), e18519. https://doi.org/10.1097/md.0000000000018519

Horvath, D. J., Li, B., Casper, T., Partida-Sanchez, S., Hunstad, D. A., Hultgren, S. J., & Justice, S. S. (2011). Morphological plasticity promotes resistance to phagocyte killing of uropathogenic Escherichia coli. *Microbes and Infection, 13*(5), 426-437. https://doi.org/10.1016/j.micinf.2010.12.004

Hossain, M., Tabassum, T., Rahman, A., Hossain, A., Afroze, T., Momen, A. M. I., Sadique, A., Sarker, M., Shams, F., Ishtiaque, A., Khaleque, A., Alam, M., Huq, A., Ahsan, G. U., & Colwell, R. R. (2020). Genotype–phenotype correlation of β-lactamase-producing uropathogenic Escherichia coli (UPEC) strains from Bangladesh. *Scientific Reports, 10*(1), 1-13. https://doi.org/10.1038/s41598-020-71213-5

Hossain, T. J. (2024). Methods for screening and evaluation of antimicrobial activity: A review of protocols, advantages, and limitations. *Eur J Microbiol Immunol (Bp), 14*(2), 97-115. https://doi.org/10.1556/1886.2024.00035

Hu, T., Chitnis, N., Monos, D., & Dinh, A. (2021). Next-generation sequencing technologies: An overview. *Human Immunology, 82*(11), 801-811. https://doi.org/10.1016/j.humimm.2021.02.012

Huntzinger, E., & Izaurralde, E. (2011). Gene silencing by microRNAs: contributions of translational repression and mRNA decay. *Nature Reviews Genetics 12*(2), 99-110. https://doi.org/10.1038/nrg2936

Huovinen, P., Sundström, L., Swedberg, G., & Sköld, O. (1995). Trimethoprim and sulfonamide resistance. *Antimicrobial Agents and Chemotherapy, 39*(2), 279-289. https://doi.org/10.1128/aac.39.2.279

Ibrahim, D. R., Dodd, C. E. R., Stekel, D. J., Meshioye, R. T., Diggle, M., Lister, M., & Hobman, J. L. (2023). Multidrug-Resistant ESBL-Producing E. coli in Clinical Samples from the UK. *Antibiotics, 12*(1), 169. https://doi.org/10.3390/antibiotics12010169

Jakovljev, A., Afset, J. E., Haugum, K., Steinum, H. O., Gresdal Rønning, T., Samuelsen, Ø., & Ås, C. G. (2022). Phenotypic and genotypic characterisation of thymine auxotrophy in Escherichia coli isolated from a patient with recurrent bloodstream infection. *PLoS ONE, 17*(7), 1-13. https://doi.org/10.1371/journal.pone.0270256

Jaureguy, F., Landraud, L., Frapy, E., Carbonnelle, E., Nassif, X., Picard, B., Passet, V., Diancourt, L., Brisse, S., Guigon, G., Lortholary, O., Clermont, O., & Denamur, E. (2008). Phylogenetic and genomic diversity of human bacteremic Escherichia coli strains. *BMC Genomics, 9*, 1-14. https://doi.org/10.1186/1471-2164-9-560

Johnson, J. R., & Russo, T. A. (2005). Molecular epidemiology of extraintestinal pathogenic (uropathogenic) Escherichia coli. *International Journal of Medical Microbiology, 295*(6/7), 383-404. https://doi.org/10.1016/j.ijmm.2005.07.005

Jones, A., Torkel, C., Stanley, D., Nasim, J., Borevitz, J., & Schwessinger, B. (2021). High-molecular weight DNA extraction, clean-up and size selection for long-read sequencing. *PLoS ONE, 16*(7), e0253830. https://doi.org/10.1371/journal.pone.0253830

Jorgensen, J. H., & Ferraro, M. J. (2009). Antimicrobial susceptibility testing: A review of general principles and contemporary practices. *Clinical Infectious Diseases, 49*(11), 1749-1755. https://doi.org/10.1086/647952

Joyce, L. F., Downes, J., Stockman, K., & Andrew, J. H. (1992). Comparison of five methods, including the PDM Epsilometer test (E test), for antimicrobial susceptibility testing of Pseudomonas aeruginosa. *J Clin Microbiol, 30*(10), 2709-2713. https://doi.org/10.1128/jcm.30.10.2709-2713.1992

Jung, J. S., Hamacher, C., Gross, B., Sparbier, K., Lange, C., Kostrzewa, M., & Schubert, S. (2016). Evaluation of a Semiquantitative Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry Method for Rapid Antimicrobial Susceptibility Testing of Positive Blood Cultures. *J Clin Microbiol, 54*(11), 2820-2824. https://doi.org/10.1128/jcm.01131-16

Justice, S. S., Hunstad, D. A., Cegelski, L., & Hultgren, S. J. (2008). Morphological plasticity as a bacterial survival strategy. *Nature Reviews Microbiology, 6*(2), 162-168. https://doi.org/10.1038/nrmicro1820

Justice, S. S., Hunstad, D. A., Seed, P. C., & Hultgren, S. J. (2006). Filamentation by Escherichia coli subverts innate defenses during urinary tract infection. *Proceedings of the National Academy of Sciences, 103*(52), 19884-19889. https://doi.org/doi:10.1073/pnas.0606329104

Kao, C.-Y., Chen, J.-W., Liu, T.-L., Yan, J.-J., & Wu, J.-J. (2018). Comparative Genomics of Escherichia coli Sequence Type 219 Clones From the Same Patient: Evolution of the IncI1 blaCMY-Carrying Plasmid in Vivo. *Frontiers in Microbiology, 9* https://doi.org/10.3389/fmicb.2018.01518

Kao, C. Y., Zhang, Y. Z., Yang, D. C., Chen, P. K., Teng, C. H., Lin, W. H., & Wang, M. C. (2023). Characterization of host and Escherichia coli strains causing recurrent urinary tract infections based on molecular typing. *BMC Microbiology, 23*(1), 1-9. https://doi.org/10.1186/s12866-023-02820-1

Kaper, J. B., Nataro, J. P., & Mobley, H. L. T. (2004). Pathogenic Escherichia coli. *Nature Reviews Microbiology, 2*(2), 123-140. https://doi.org/10.1038/nrmicro818

Kapoor, G., Saigal, S., & Elongavan, A. (2017). Action and resistance mechanisms of antibiotics: A guide for clinicians. *J Anaesthesiol Clin Pharmacol, 33*(3), 300-305. https://doi.org/10.4103/joacp.JOACP_349_15

Karami, A., Mazloom Zadeh, S., Rastin, A., Karami, A., & Shiri, P. (2015). The consistency of antibiotic resistance' results in two methods of disk diffusion and MIC in isolated organisms from patients with urinary tract infections. *J Med Life, 8*(Spec Iss 4), 282-286.

Khanal, N., Cortie, C. H., Story, C., Jones, S., Mansfield, K. J., Miyakis, S., & Keighley, C. (2024). Multidrug resistance in urinary E. coli higher in males compared to females. *BMC Urology, 24*(1), 255. https://doi.org/10.1186/s12894-024-01654-x

Kim, Y. H., Park, J., & Chung, H. S. (2023). Genetic characterization of tetracycline-resistant Staphylococcus aureus with reduced vancomycin susceptibility using whole-genome sequencing. *206*(1), 1-10. https://doi.org/10.1007/s00203-023-03760-0

Klein, R. D., & Hultgren, S. J. (2020). Urinary tract infections: microbial pathogenesis, host-pathogen interactions and new treatment strategies. *Nature Reviews Microbiology, 18*(4), 211-226. https://doi.org/10.1038/s41579-020-0324-0

Kordus, S. L., & Baughn, A. D. (2019). Revitalizing antifolates through understanding mechanisms that govern susceptibility and resistance. *Med. Chem. Commun., 10*(6), 880-895. https://doi.org/10.1039/c9md00078j

Kot, B. (2019). Antibiotic Resistance Among Uropathogenic Escherichia coli. *Polish Journal of Microbiology, 68*(4), 403-415. https://doi.org/10.33073/pjm-2019-048

Koudounas, S., Minematsu, T., Mugita, Y., Tomida, S., Nakai, A., Nakagami, G., & Sanada, H. (2021). Inhibiting effect of synthetic urine on the growth and mobility of Pseudomonas aeruginosa, Escherichia coli, Proteus mirabilis and Klebsiella pneumoniae shifts to a promoting effect during culture. *Journal of Nursing Science and Engineering, 8*, 220-229. https://doi.org/10.24462/jnse.8.0_220

Kowalska-Krochmal, B., & Dudek-Wicher, R. (2021). The minimum inhibitory concentration of antibiotics: Methods, interpretation, clinical relevance. *Pathogens, 10*(2), 1-21. https://doi.org/10.3390/pathogens10020165

Krause, K. M., Serio, A. W., Kane, T. R., & Connolly, L. E. (2016). Aminoglycosides: An overview. *Cold Spring Harb Perspect Med, 6*(6), 1-18. https://doi.org/10.1101/cshperspect.a027029

Krishnamurthi, V. R., Niyonshuti, I. I., Chen, J., & Wang, Y. (2021). A new analysis method for evaluating bacterial growth with microplate readers. *PLoS ONE, 16*(1), e0245205. https://doi.org/10.1371/journal.pone.0245205

Krucinska, J., Lombardo, M. N., Erlandsen, H., Estrada, A., Si, D., Viswanathan, K., & Wright, D. L. (2022). Structure-guided functional studies of plasmid-encoded dihydrofolate reductases reveal a common mechanism of trimethoprim resistance in Gram-negative pathogens. *Communications Biology, 5*(1), 1-14. https://doi.org/10.1038/s42003-022-03384-y

Kukurba, K. R., & Montgomery, S. B. (2015). RNA sequencing and analysis. *Cold Spring Harbor Protocols, 11*, 951-969. https://doi.org/10.1101/pdb.top084970

Labar, A. S., Millman, J. S., Ruebush, E., Opintan, J. A., Bishar, R. A., Aboderin, A. O., Newman, M. J., Lamikanra, A., & Okeke, I. N. (2012). Regional Dissemination of a Trimethoprim-Resistance Gene Cassette via a Successful Transposable Element. *PLoS ONE, 7*(5), e38142. https://doi.org/10.1371/journal.pone.0038142

LabPLUS. (2023). *Antibiotic susceptibility reports*. [Accessed on 13 August 2023] from http://www.labplus.co.nz/clinical-resources/antibiotic-susceptibility-reports/

Lacotte, Y., Ploy, M.-C., & Raherison, S. (2017). Class 1 integrons are low-cost structures in Escherichia coli. *The ISME Journal, 11*(7), 1535-1544. https://doi.org/10.1038/ismej.2017.38

Lammers, R. L., Gibson, S., Kovacs, D., Sears, W., & Strachan, G. (2001). Comparison of test characteristics of urine dipstick and urinalysis at various test cutoff points. *Annals of Emergency Medicine, 38*(5), 505-512. https://doi.org/10.1067/mem.2001.119427

Lange, C., Schubert, S., Jung, J., Kostrzewa, M., & Sparbier, K. (2014). Quantitative Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry for Rapid Resistance Detection. *Journal of Clinical Microbiology, 52*(12), 4155-4162. https://doi.org/doi:10.1128/jcm.01872-14

Lara, F. B. M., Pereira, A. L., Nery, D. R., de Oliveira, P. M., Araujo, M. L., Carvalho, F. R. Q., Messias-Silva, L. C. F., Ferreira, L. B., & Faria-Junior, C. (2017). Virulence markers and phylogenetic analysis of Escherichia coli strains with hybrid EAEC/UPEC genotypes recovered from sporadic cases of extraintestinal infections. *Frontiers in Microbiology, 8*, 1-11. https://doi.org/10.3389/fmicb.2017.00146

Lauxen, A. I., Kobauri, P., Wegener, M., Hansen, M., J. , Galenkamp, N., S., Maglia, G., Szymanski, W., Feringa, B., L. , & Kuipers, O., P. (2021). Mechanism of resistance development in E. coli against TCAT, a trimethoprim-based photoswitchable antibiotic. *Pharamaceuticals, 14*(392), 1-18. https://doi.org/10.3390/ph14050392

Le, T. P., & Miller, L. G. (2001). Empirical therapy for uncomplicated urinary tract infections in an era of increasing antimicrobial resistance: A decision and cost analysis. *Clinical Infectious Diseases, 33*(5), 615-621. https://doi.org/10.1086/322603

Lee, C. H. J., Norris, P., Duck, I. M., & Sibley, C. G. (2018). Demographic and psychological factors associated with feelings of antibiotic entitlement in New Zealand. *Antibiotics, 7*(3), 1-14. https://doi.org/10.3390/antibiotics7030082

Lee, D. S., Lee, S. J., & Choe, H. S. (2018a). Community-acquired urinary tract infection by Escherichia coli in the era of antibiotic resistance. *BioMed Research International, 2018*, 1-15. https://doi.org/10.1155/2018/7656752

Lee, D. S., Lee, S. J., & Choe, H. S. (2018b). Community-acquired urinary tract infection by Escherichia coli in the era of antibiotic resistance. *BioMed Research International, 2018*. https://doi.org/10.1155/2018/7656752

Lee, H., & Ko, K. S. (2021). Effect of multiple, compatible plasmids on the fitness of the bacterial host by inducing transcriptional changes. *Journal of Antimicrobial Chemotherapy (JAC), 76*(10), 2528-2537. https://doi.org/10.1093/jac/dkab240

Lee, J. C., Oh, J. Y., Cho, J. W., Park, J. C., Kim, J. M., Seol, S. Y., & Cho, D. T. (2001). The prevalence of trimethoprim-resistance-conferring dihydrofolate reductase genes in urinary isolates of Escherichia coli in Korea. *J Antimicrob Chemother, 47*(5), 599-604. https://doi.org/10.1093/jac/47.5.599

Leimbach, A., Hacker, J., & Dobrindt, U. (2013). E. coli as an all-rounder: the thin line between commensalism and pathogenicity. *Current Topics in Microbiology and Immunology, 358*, 3-32. https://doi.org/10.1007/82_2012_303

Lemay-St-Denis, C., Diwan, S.-S., & Pelletier, J. N. (2021). The bacterial genomic context of highly trimethoprim-resistant DfrB dihydrofolate reductases highlights an emerging threat to public health. *Antibiotics, 10*(4), 1-13. https://doi.org/10.3390/antibiotics10040433

Levison, M. E., & Levison, J. H. (2009). Pharmacokinetics and pharmacodynamics of antibacterial agents. *Infectious Disease Clinics of North America 23*(4), 791-815. https://doi.org/10.1016/j.idc.2009.06.008

Lewnard, J. A., Charani, E., Gleason, A., Hsu, L. Y., Khan, W. A., Karkey, A., Chandler, C. I. R., Mashe, T., Khan, E. A., Bulabula, A. N. H., Donado-Godoy, P., & Laxminarayan, R. (2024). Burden of bacterial antimicrobial resistance in low-income and middle-income countries avertible by existing interventions: an evidence review and modelling analysis. *Lancet, 403*(10442), 2439-2454. https://doi.org/10.1016/s0140-6736(24)00862-6

Li, B., & Dewey, C. N. (2011). RSEM: Accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics, 12*, 1-16. https://doi.org/10.1186/1471-2105-12-323

Li, D., Reid, C. J., Kudinha, T., Jarocki, V. M., & Djordjevic, S. P. (2020). Genomic analysis of trimethoprim-resistant extraintestinal pathogenic Escherichia coli and recurrent urinary tract infections. *Microbial Genomics, 6*(12), 1-19. https://doi.org/10.1099/mgen.0.000475

Li, X. Z., & Nikaido, H. (2009). Efflux-mediated drug resistance in bacteria: an update. *Drugs, 69*(12), 1555-1623. https://doi.org/10.2165/11317030-000000000-00000

Lipworth, S., Crook, D., Walker, A. S., Peto, T., & Stoesser, N. (2024). Exploring uncatalogued genetic variation in antimicrobial resistance gene families in Escherichia coli: an observational analysis. *The Lancet Microbe, 5*(11), 1-11. https://doi.org/10.1016/S2666-5247(24)00152-6

Lloyd, A. L., Henderson, T. A., Vigil, P. D., & Mobley, H. L. (2009). Genomic islands of uropathogenic Escherichia coli contribute to virulence. *Journal of Bacteriology, 191*(11), 3469-3481. https://doi.org/10.1128/jb.01717-08

Lohner, K., & Blondelle, S. E. (2005). Molecular mechanisms of membrane perturbation by antimicrobial peptides and the use of biophysical studies in the design of novel peptide antibiotics. *Comb Chem High Throughput Screen, 8*(3), 241-256. https://doi.org/10.2174/1386207053764576

Lopatkin, A. J., Meredith, H. R., Srimani, J. K., Pfeiffer, C., Durrett, R., & You, L. (2017). Persistence and reversal of plasmid-mediated antibiotic resistance. *Nature Communications, 8*(1), 1689. https://doi.org/10.1038/s41467-017-01532-1

Luna-Pineda, V. M., Reyes-Grajeda, J. P., Cruz-Córdova, A., Saldaña-Ahuactzi, Z., Ochoa, S. A., Maldonado-Bernal, C., Cázares-Domínguez, V., Moreno-Fierros, L., Arellano-Galindo, J., Hernández-Castro, R., & Xicohtencatl-Cortes, J. (2016). Dimeric and trimeric fusion proteins generated with fimbrial adhesins of uropathogenic Escherichia coli. *Frontiers in Cellular & Infection Microbiology, 6*, 1-15. https://doi.org/10.3389/fcimb.2016.00135

Luscombe, N. M., Greenbaum, D., & Gerstein, M. (2001). What is bioinformatics? A proposed definition and overview of the field. *Methods of Information in Medicine, 40*(4), 346-358.

MacGowan, A. P., & Wise, R. (2001). Establishing MIC breakpoints and the interpretation of in vitro susceptibility tests. *Journal of Antimicrobial Chemotherapy, 48*, 17-28. https://doi.org/10.1093/jac/48.suppl_1.17

Mangin, D., Toop, L., Chambers, S., Ikram, R. B., & Harris, B. H. (2005). Increased rates of trimethoprim resistance in uncomplicated urinary tract infection: Cause for concern? *New Zealand medical journal, 118*(1225). [Accessed on 21 January 2022] from https://pubmed.ncbi.nlm.nih.gov/16286940/

Mann, R., Mediati, D. G., Duggin, I. G., Harry, E. J., & Bottomley, A. L. (2017). Metabolic adaptations of uropathogenic E. coli in the urinary tract. *Frontiers in Cellular and Infection Microbiology, 7*, 1-15. https://doi.org/10.3389/fcimb.2017.00241

Manna, M. S., Tamer, Y. T., Gaszek, I., Poulides, N., Ahmed, A., Wang, X., Toprak, F. C. R., Woodard, D. R., Koh, A. Y., Williams, N. S., Borek, D., Atilgan, A. R., Hulleman, J. D., Atilgan, C., Tambar, U., & Toprak, E. (2021). A trimethoprim derivative impedes antibiotic resistance evolution. *Nature Communications 12*(1), 1-10. https://doi.org/10.1038/s41467-021-23191-z

Manoharan-Basil, S. S., Balduck, M., Gestels, Z., Abdellati, S., Block, T. D., & Kenyon, C. (2025). Transcriptomic profiling of ceftriaxone-tolerant phenotypes of Neisseria gonorrhoeae reveals downregulation of ribosomal genes 2014; a pilot study. *Microbiology Spectrum, 13*(8), e01207-01225. https://doi.org/10.1128/spectrum.01207-25

Marques, A. G., Doi, A. M., Pasternak, J., Damascena, M. D. S., Martino, M. D. V., & França, C. N. (2017). Performance of the dipstick screening test as a predictor of negative urine culture. *Einstein (Sao Paulo, Brazil), 15*(1), 34-39. https://doi.org/10.1590/S1679-45082017AO3936

Martínez-Figueroa, C., Cortés-Sarabia, K., del Carmen Alarcón-Romero, L., Catalán-Nájera, H. G., Martínez-Alarcón, M., & Vences-Velázquez, A. (2020). Observation of intracellular bacterial communities in urinary sediment using brightfield microscopy; a case report. *BMC Urology, 20*(1), 1-4. https://doi.org/10.1186/s12894-020-00661-y

Maskell, R., Okubadejo, O. A., Payne, R. H., & Pead, L. (1978). Human infections with thymine-requiring bacteria. *J Med Microbiol., 11*(1), 33-45. https://doi.org/10.1099/00222615-11-1-33

Mathieu, A., Fleurier, S., Frénoy, A., Bredeche, M. F., Sanchez-Vizuete, P., Song, X., Matic, I., & Dairou, J. (2016). Discovery and function of a general core hormetic stress response in E. coli induced by sublethal concentrations of antibiotics. *Cell Reports, 17*(1), 46-57. https://doi.org/10.1016/j.celrep.2016.09.001

Matos, O., & Esteves, F. (2010). Epidemiology and clinical relevance of Pneumocystis jirovecii Frenkel, 1976 dihydropteroate synthase gene mutations. *Parasite, 17*(3), 219-232. https://doi.org/10.1051/parasite/2010173219

Matuschek, E., Brown, D. F., & Kahlmeter, G. (2014). Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. *Clinical Microbiology and Infection, 20*(4), 255-266. https://doi.org/10.1111/1469-0691.12373

Maxson, T., Taylor-Howell, C. L., & Minogue, T. D. (2017). Semi-quantitative MALDI-TOF for antimicrobial susceptibility testing in Staphylococcus aureus. *PLoS ONE, 12*(8), e0183899. https://doi.org/10.1371/journal.pone.0183899

Mazurek, J., Bok, E., Stosik, M., & Baldy-Chudzik, K. (2015). Antimicrobial Resistance in Commensal Escherichia coli from Pigs during Metaphylactic Trimethoprim and Sulfamethoxazole Treatment and in the Post-Exposure Period. *International Journal of Environmental Research and Public Health, 12*(2), 2150-2163. https://doi.org/10.3390/ijerph120202150

McArthur, A. G., Waglechner, N., Nizam, F., Yan, A., Azad, M. A., Baylay, A. J., Bhullar, K., Canova, M. J., Pascale, G. D., Ejim, L., Kalan, L., King, A. M., Koteva, K., Morar, M., Mulvey, M. R., O'Brien, J. S., Pawlowski, A. C., Piddock, L. J. V., Spanogiannopoulos, P., Sutherland, A. D., Tang, I., Taylor, P. L., Thaker, M., Wang, W., Yan, M., Yu, T., & Wright, G. D. (2013). The Comprehensive Antibiotic Resistance Database. *Antimicrobial Agents and Chemotherapy, 57*(7), 3348-3357. https://doi.org/doi:10.1128/aac.00419-13

McCormick, J. Z., Cardwell, S. M., Wheelock, C., Wong, C. M., & Vander Weide, L. A. (2020). Impact of ambulatory antimicrobial stewardship on prescribing patterns for urinary tract infections. *Journal of Clinical Pharmacy & Therapeutics, 45*(6), 1312-1319. https://doi.org/10.1111/jcpt.13210

Medina, M., & Castillo-Pino, E. (2019). An introduction to the epidemiology and burden of urinary tract infections. *Therapeutic Advances in Urology, 11*, 3-7. https://doi.org/10.1177/1756287219832172

Melnyk, A. H., Kassen, R., & Wong, A. (2015). The fitness costs of antibiotic resistance mutations. *Evolutionary Applications, 8*(3), 273-283. https://doi.org/10.1111/eva.12196

Minato, Y., Dawadi, S., Kordus, S. L., Sivanandam, A., Aldrich, C. C., & Baughn, A. D. (2018). Mutual potentiation drives synergy between trimethoprim and sulfamethoxazole. *Nature Communications, 9*(1), 1003. https://doi.org/10.1038/s41467-018-03447-x

Ministry of Health. (2023). *Together we can keep antimicrobials working*. [Accessed on 31 August 2023] from https://www.health.govt.nz/our-work/diseases-and-conditions/antimicrobial-resistance/together-we-can-keep-antimicrobials-working

Mira, P., Yeh, P., & Hall, B. G. (2022). Estimating microbial population data from optical density. *PLoS ONE, 17*(10), 1-8. https://doi.org/10.1371/journal.pone.0276040

Mohapatra, S., Ghosh, D., Vivekanandan, P., Chunchanur, S., Venugopal, S., Tak, V., Panigrahy, R., Chaudhuri, S., Pundir, S., Sharma, T., Kocher, D., Singh, H., Gautam, H., Sood, S., Das, B. K., Kapil, A., Kumar, A., Kumari, R., Kalaivani, M., Rangaiah, A., Salve, H. R., Malhotra, S., Kant, S., Hari, P., Mohapatra, S., Sneha, K. C., Shwetha, J. V., Tak, V., Panigrahy, R., & Chaudhuri, S. (2023). Genome profiling of uropathogenic E. coli from strictly defined community-acquired UTI in paediatric patients: a multicentric study. *Antimicrobial Resistance & Infection Control, 12*(1), 1-16. https://doi.org/10.1186/s13756-023-01233-z

Möller, J., Emge, P., Vizcarra, I. A., Kollmannsberger, P., & Vogel, V. (2013). Bacterial filamentation accelerates colonization of adhesive spots embedded in biopassive surfaces. *New Journal of Physics, 15*(12), 125016-125034. https://doi.org/10.1088/1367-2630/15/12/125016

Morè, N., Martorana, A. M., Biboy, J., Otten, C., Winkle, M., Serrano, C. K. G., Silva, A. M., Atkinson, L., Yau, H., Breukink, E., Blaauwen, T. d., Vollmer, W., & Polissi, A. (2019). Peptidoglycan Remodeling Enables Escherichia coli To Survive Severe Outer Membrane Assembly Defect. *mBio, 10*(1), 1-18. https://doi.org/10.1128/mbio.02729-18

Mulvey, M. A., Schilling, J. D., Martinez, J. J., & Hultgren, S. J. (2000). Bad bugs and beleaguered bladders: Interplay between uropathogenic Escherichia coli and innate host defenses. *Proceedings of the National Academy of Sciences of the United States of America, 97*(16), 8829-8835. https://doi.org/10.1073/pnas.97.16.8829

Munita, J. M., & Arias, C. A. (2016). Mechanisms of antibiotic resistance. *Microbiology Spectrum, 4*(2). https://doi.org/10.1128/microbiolspec.VMBF-0016-2015

Murray, B. E., Alvarado, T., Kim, K. H., Vorachit, M., Jayanetra, P., Levine, M. M., Prenzel, I., Fling, M., Elwell, L., McCracken, G. H., & et al. (1985). Increasing resistance to trimethoprim-sulfamethoxazole among isolates of Escherichia coli in developing countries. *J Infect Dis, 152*(6), 1107-1113. https://doi.org/10.1093/infdis/152.6.1107

Murray, B. E., Rensimer, E. R., & Dupont, H. L. (1982). Emergence of High-Level Trimethoprim Resistance in Fecal Escherichia coli during Oral Administration of Trimethoprim or Trimethoprim-Sulfamethoxazole. *306*(3), 130-135. https://doi.org/10.1056/NEJM198201213060302

Murray, B. O., Flores, C., Williams, C., Flusberg, D. A., Marr, E. E., Kwiatkowska, K. M., Charest, J. L., Isenberg, B. C., & Rohn, J. L. (2021). Recurrent urinary tract infection: A mystery in search of better model systems. *Frontiers in Cellular & Infection Microbiology, 11*, 1-29. https://doi.org/10.3389/fcimb.2021.691210

Myoda, T. T., Lowther, S. V., Funanage, V. L., & Young, F. E. (1984). Cloning and mapping of the dihydrofolate reductase gene of Bacillus subtilis. *Gene, 29*(1), 135-143. https://doi.org/10.1016/0378-1119(84)90174-4

Nakamura, M., Tomoda, Y., Kobayashi, M., Hanaki, H., & Kanoh, Y. (2025). Sulfamethoxazole-trimethoprim plus rifampicin combination therapy for methicillin-resistant Staphylococcus aureus infection: An in vitro study. *PLoS ONE, 20*(5), e0323935. https://doi.org/10.1371/journal.pone.0323935

Nan, J., Brostromer, E., Liu, X.-Y., Kristensen, O., & Su, X.-D. (2009). Bioinformatics and Structural Characterization of a Hypothetical Protein from Streptococcus mutans: Implication of Antibiotic Resistance. *PLoS ONE, 4*(10), e7245. https://doi.org/10.1371/journal.pone.0007245

Nasrollahian, S., Graham, J. P., & Halaji, M. (2024). A review of the mechanisms that confer antibiotic resistance in pathotypes of E. coli [Review]. *Frontiers in Cellular and Infection Microbiology, Volume 14 - 2024*. https://doi.org/10.3389/fcimb.2024.1387497

Ndagi, U., Falaki, A. A., Abdullahi, M., Lawal, M. M., & Soliman, M. E. (2020). Antibiotic resistance: Bioinformatics-based understanding as a functional strategy for drug design. *Royal Society of Chemistry Advances, 10*(31), 18451-18468. https://doi.org/10.1039/d0ra01484b

Nemeth, J., Oesch, G., & Kuster, S. P. (2014). Bacteriostatic versus bactericidal antibiotics for patients with serious bacterial infections: systematic review and meta-analysis. *Journal of Antimicrobial Chemotherapy, 70*(2), 382-395. https://doi.org/10.1093/jac/dku379

New Zealand Formulary. (2023). *Urinary-Tract Infection*. Retrieved August 07 2023 from https://nzf.org.nz/nzf_71068

Nickel, J. C. (2005). Practical management of recurrent urinary tract infections in premenopausal women. *Reviews in Urology, 7*(1), 11-17.

Nicolle, L. E., Gupta, K., Bradley, S. F., Colgan, R., DeMuri, G. P., Drekonja, D., Eckert, L. O., Geerlings, S. E., Köves, B., Hooton, T. M., Juthani-Mehta, M., Knight, S. L., Saint, S., Schaeffer, A. J., Trautner, B., Wullt, B., & Siemieniuk, R. (2019). Clinical Practice Guideline for the Management of Asymptomatic Bacteriuria: 2019 Update by the Infectious Diseases Society of Americaa. *Clinical Infectious Diseases, 68*(10), e83-e110. https://doi.org/10.1093/cid/ciy1121

Nielsen, K. L., Dynesen, P., Larsen, P., & Frimodt-Møller, N. (2014). Faecal Escherichia coli from patients with E. coli urinary tract infection and healthy controls who have never had a urinary tract infection. *Journal of Medical Microbiology, 63*(4), 582-589. https://doi.org/10.1099/jmm.0.068783-0

Nolan, L. K., Li, G., & Logue, C. M. (2015). Origin and Dissemination of Antimicrobial Resistance among Uropathogenic Escherichia coli. *Microbiology Spectrum, 3*(5), 10.1128/microbiolspec.uti-0007-2012. https://doi.org/10.1128/microbiolspec.uti-0007-2012

Novelli, A., & Rosi, E. (2017). Pharmacological properties of oral antibiotics for the treatment of uncomplicated urinary tract infections. *Journal of Chemotherapy, 29*, 10-18. https://doi.org/10.1080/1120009X.2017.1380357

Novelli, M., & Bolla, J.-M. (2024). RND Efflux Pump Induction: A Crucial Network Unveiling Adaptive Antibiotic Resistance Mechanisms of Gram-Negative Bacteria. *Antibiotics, 13*(6), 1-21. https://doi.org/10.3390/antibiotics13060501

O'Grady, M. C., Barry, L., Corcoran, G. D., Hooton, C., Sleator, R. D., & Lucey, B. (2018). Empirical treatment of urinary tract infections: How rational are our guidelines? *Journal of Antimicrobial Chemotherapy, 74*(1), 214-217. https://doi.org/10.1093/jac/dky405

Otoupal, P. B., Eller, K. A., Erickson, K. E., Campos, J., Aunins, T. R., & Chatterjee, A. (2021). Potentiating antibiotic efficacy via perturbation of non-essential gene expression. *Communications Biology, 4*(1), 1-15. https://doi.org/10.1038/s42003-021-02783-x

Paganini, J. A., Plantinga, N. L., Arredondo-Alonso, S., Willems, R. J. L., & Schürch, A. C. (2021). Recovering Escherichia coli Plasmids in the Absence of Long-Read Sequencing Data. *Microorganisms, 9*(8), 1613. https://doi.org/10.3390/microorganisms9081613

Partridge, S. R., Kwong, S. M., Firth, N., & Jensen, S. O. (2018). Mobile Genetic Elements Associated with Antimicrobial Resistance. *Clinical Microbiology Reviews, 31*(4), 1-61. https://doi.org/10.1128/cmr.00088-17

Pato, M. L., & Brown, G. M. (1963). Mechanisms of resistance of Escherichia coli to sulfonamides. *Archives of Biochemistry and Biophysics, 103*(3), 443-448. https://doi.org/10.1016/0003-9861(63)90435-1

Pattis, I., Weaver, L., Burgess, S., Ussher, J., E. , & Kristin, D. (2022). Antimicrobial resistance in New Zealand - A one health perspective. *Antibiotics, 11*(778), 1-26. https://doi.org/10.3390/antibiotics11060778

Peng, Z., Hayen, A., Hall, J., & Liu, B. (2021). Microbiology testing and antibiotic treatment for urinary tract infections in general practice: a nationwide observational study. *Infection, 49*(2), 249-255. https://doi.org/10.1007/s15010-020-01512-6

Pierce, V. M., Bhowmick, T., & Simner, P. J. (2023). Guiding antimicrobial stewardship through thoughtful antimicrobial susceptibility testing and reporting strategies: an updated approach in 2023. *Journal of Clinical Microbiology, 61*(11), e00074-00022. https://doi.org/doi:10.1128/jcm.00074-22

Podnecky, N. L., Rhodes, K. A., Mima, T., Drew, H. R., Chirakul, S., Wuthiekanun, V., Schupp, J. M., Sarovich, D. S., Currie, B. J., Keim, P., & Schweizer, H. P. (2017). Mechanisms of Resistance to Folate Pathway Inhibitors in Burkholderia pseudomallei: Deviation from the Norm. *mBio, 8*(5), 1-18. https://doi.org/10.1128/mbio.01357-17

Poey, M. E., de los Santos, E., Aznarez, D., García-Laviña, C. X., & Laviña, M. (2024). Genetics of resistance to trimethoprim in cotrimoxazole resistant uropathogenic Escherichia coli: integrons, transposons, and single gene cassettes. *Frontiers in Microbiology, 15*, 1-13. https://doi.org/10.3389/fmicb.2024.1395953

Poonawala, H., Zhang, Y., Kuchibhotla, S., Green, A. G., Cirillo, D. M., Marco, F. D., Spitlaeri, A., Miotto, P., & Farhat, M. R. (2024). Transcriptomic responses to antibiotic exposure in Mycobacterium tuberculosis. *Antimicrobial Agents and Chemotherapy, 68*(5), e01185-01123. https://doi.org/10.1128/aac.01185-23

Pujades-Rodriguez, M., West, R. M., Wilcox, M. H., & Sandoe, J. (2019). Lower urinary tract infections: Management, outcomes and risk factors for antibiotic re-prescription in primary care. *EClinicalMedicine, 14*, 23-31. https://doi.org/10.1016/j.eclinm.2019.07.012

Qin, H., Loo, J. F. C., Lin, X., Yim, A. K. Y., Chan, T. F., Lo, N. W. S., Ip, M., Tsui, S. K. W., & Lau, T. C. K. (2018). Comparative transcriptomics of multidrug-resistant Acinetobacter baumannii in response to antibiotic treatments. *Scientific Reports, 8*(1). https://doi.org/10.1038/s41598-018-21841-9

R Core Team. (2021). *R: A language and environment for statistical computing.* https://www.R-project.org/

Raheem, N., & Straus, S. K. (2019). Mechanisms of Action for Antimicrobial Peptides With Antibacterial and Antibiofilm Functions. *Frontiers in Microbiology, Volume 10 - 2019*. https://doi.org/10.3389/fmicb.2019.02866

Ranjbar, R., Nazari, S., & Farahani, O. (2020). Phylogenetic Analysis and Antimicrobial Resistance Profiles of Escherichia coli Strains Isolated from UTI-Suspected Patients. *Iranian Journal of Public Health, 49*(9), 1743-1749. https://doi.org/10.18502/ijph.v49i9.4094

Rasko, D. A., Rosovitz, M. J., Myers, G. S., Mongodin, E. F., Fricke, W. F., Gajer, P., Crabtree, J., Sebaihia, M., Thomson, N. R., Chaudhuri, R., Henderson, I. R., Sperandio, V., & Ravel, J. (2008). The pangenome structure of Escherichia coli: comparative genomic analysis of E. coli commensal and pathogenic isolates. *Journal of Bacteriology, 190*(20), 6881-6893. https://doi.org/10.1128/jb.00619-08

Ray, B., & Speck, M. L. (1973). Freeze-Injury in Bacteria. *CRC Critical Reviews in Clinical Laboratory Sciences, 4*(2), 161-213. https://doi.org/10.3109/10408367309151556

Rode, C. K., Melkerson-Watson, L. J., Johnson, A. T., & Bloch, C. A. (1999). Type-specific contributions to chromosome size differences in Escherichia coli. *Infection and Immunology, 67*(1), 230-236. https://doi.org/10.1128/iai.67.1.230-236.1999

Rosen, D. A., Hooton, T. M., Stamm, W. E., Humphrey, P. A., & Hultgren, S. J. (2007). Detection of intracellular bacterial communities in human urinary tract infection. *PLoS Medicine, 4*(12), 1949-1958. https://doi.org/10.1371/journal.pmed.0040329

Rouli, L., Merhej, V., Fournier, P. E., & Raoult, D. (2015). The bacterial pangenome as a new tool for analysing pathogenic bacteria. *New Microbes and New Infections, 7*, 72-85. https://doi.org/10.1016/j.nmni.2015.06.005

Rozwadowski, M., & Gawel, D. (2022). Molecular factors and mechanisms driving multidrug resistance in uropathogenic Escherichia coli - An update. *Genes, 13*(8), 1-19. https://doi.org/10.3390/genes13081397

Ruța, F., Pribac, M., Mardale, E., Suciu, S., Maior, R., Bogdan, S., & Avram, C. (2024). Associations between Gut Microbiota Dysbiosis and Other Risk Factors in Women with a History of Urinary Tract Infections. *Nutrients, 16*(11), 1-15. https://doi.org/10.3390/nu16111753

Saeb, A. T., Abouelhoda, M., Selvaraju, M., Althawadi, S. I., Mutabagani, M., Adil, M., Al Hokail, A., & Tayeb, H. T. (2017). The use of next-generation sequencing in the identification of a fastidious pathogen: a lesson from a clinical setup. *Evol Bioinform Online, 12*, 1176934316686072. https://doi.org/10.1177/1176934316686072

Salam, M. A., Al-Amin, M. Y., Salam, M. T., Pawar, J. S., Akhter, N., Rabaan, A. A., & Alqumber, M. A. A. (2023). Antimicrobial Resistance: A Growing Serious Threat for Global Public Health. *Healthcare, 11*(13), 1-20. https://www.mdpi.com/2227-9032/11/13/1946

Sánchez-Osuna, M., Cortés, P., Llagostera, M., Barbé, J., & Erill, I. (2020). Exploration into the origins and mobilization of di-hydrofolate reductase genes and the emergence of clinical resistance to trimethoprim. *Microbial Genomics, 6*(11), 1-13. https://doi.org/10.1099/mgen.0.000440

Sangurdekar, D. P., Khodursky, A. B., & Zhang, Z. (2011). The association of DNA damage response and nucleotide level modulation with the antibacterial mechanism of the anti-folate drug trimethoprim. *BMC Genomics, 12*, 1-14. https://doi.org/10.1186/1471-2164-12-583

Santana, A. P., Korn, L., Betsch, C., Krockow, E. M., Sievert, E. D. C., Gross, M., Pepper, M., & Böhm, R. (2024). Understanding and reducing inappropriate antibiotic use in the context of delayed prescriptions. *Health Psychol, 43*(3), 194-202. https://doi.org/10.1037/hea0001323

Santos, A. C. d. M., Santos, F. F., Silva, R. M., & Gomes, T. A. T. (2020). Diversity of hybrid- and hetero-pathogenic Escherichia coli and their potential implication in more severe diseases. *Frontiers in Cellular and Infection Microbiology, 10*, 1-11. https://doi.org/10.3389/fcimb.2020.00339

Sarowska, J., Futoma-Koloch, B., Jama-Kmiecik, A., Frej-Madrzak, M., Ksiazczyk, M., Bugla-Ploskonska, G., & Choroszy-Krol, I. (2019). Virulence factors, prevalence and potential transmission of extraintestinal pathogenic Escherichia coli isolated from different sources: Recent reports. *Gut Pathogens, 11*(1), 1-16. https://doi.org/10.1186/s13099-019-0290-0

Sarshar, M., Scribano, D., Limongi, D., Zagaglia, C., Palamara, A. T., & Ambrosi, C. (2022). Adaptive strategies of uropathogenic Escherichia coli CFT073: from growth in lab media to virulence during host cell adhesion. *International microbiology : the official journal of the Spanish Society for Microbiology, 25*(3), 481-494. https://doi.org/10.1007/s10123-022-00235-y

Schito, G. C., Gualco, L., Marchese, A., Naber, K. G., Botto, H., Palou, J., & Mazzei, T. (2009). The ARESC study: An international survey on the antimicrobial resistance of pathogens involved in uncomplicated urinary tract infections. *International Journal of Antimicrobial Agents, 34*(5), 407-413. https://doi.org/10.1016/j.ijantimicag.2009.04.012

Schmidt, K., Stanley, K. K., Hale, R., Smith, L., Wain, J., O'Grady, J., & Livermore, D. M. (2019). Evaluation of multiplex tandem PCR (MT-PCR) assays for the detection of bacterial resistance genes among Enterobacteriaceae in clinical urines. *Journal of Antimicrobial Chemotherapy (JAC), 74*(2), 349-356. https://doi.org/10.1093/jac/dky419

Schneeberger, C., Kazemier, B. M., & Geerlings, S. E. (2014). Asymptomatic bacteriuria and urinary tract infections in special patient groups: women with diabetes mellitus and pregnant women. *Current Opinion in Infectious Diseases 27*(1), 108-114. https://doi.org/10.1097/QCO.0000000000000028

Schober, A. F., Mathis, A. D., Ingle, C., Reynolds, K. A., Park, J. O., Chen, L., Rabinowitz, J. D., Junier, I., & Rivoire, O. (2019). A two-enzyme adaptive unit within bacterial folate metabolism. *Cell Reports, 27*(11), 3359-3370. https://doi.org/10.1016/j.celrep.2019.05.030

Schreiber, H. L. t., Conover, M. S., Chou, W. C., Hibbing, M. E., Manson, A. L., Dodson, K. W., Hannan, T. J., Roberts, P. L., Stapleton, A. E., Hooton, T. M., Livny, J., Earl, A. M., & Hultgren, S. J. (2017). Bacterial virulence phenotypes of Escherichia coli and host susceptibility determine risk for urinary tract infections. *Science Translation Medicine, 9*(382), 1-31. https://doi.org/10.1126/scitranslmed.aaf1283

Seoane, A., & Bou, G. (2021). Bioinformatics approaches to the study of antimicrobial resistance. *Rev Esp Quimioter, 34 Suppl 1*(Suppl1), 15-17. https://doi.org/10.37201/req/s01.04.2021

Šeputienė, V., Povilonis, J., Ružauskas, M., Pavilonis, A., & Sužiedėlienė, E. (2010). Prevalence of trimethoprim resistance genes in Escherichia coli isolates of human and animal origin in Lithuania. *Journal of Medical Microbiology, 59*(3), 315-322. https://doi.org/10.1099/jmm.0.015008-0

Sharma, K., Dhar, N., Thacker, V. V., Simonet, T. M., Signorino-Gelo, F., Knott, G. W., & McKinney, J. D. (2021). Dynamic persistence of UPEC intracellular bacterial communities in a human bladderchip model of urinary tract infection. *eLife*, 1-30. https://doi.org/10.7554/eLife.66481

Sharma, P., Haycocks, J. R. J., Middlemiss, A. D., Kettles, R. A., Sellars, L. E., Ricci, V., Piddock, L. J. V., & Grainger, D. C. (2017). The multiple antibiotic resistance operon of enteric bacteria controls DNA repair and outer membrane integrity. *Nature Communications, 8*(1), 1-12. https://doi.org/10.1038/s41467-017-01405-7

Shea, A. E., Frick-Cheng, A. E., Smith, S. N., & Mobley, H. L. T. (2022). Phenotypic assessment of clinical Escherichia coli isolates as an indicator for uropathogenic potential. *MSystems, 7*(6), 1-17. https://doi.org/10.1128/msystems.00827-22

Sheikh, A. F., Goodarzi, H., Yadyad, M. J., Aslani, S., Amin, M., Jomehzadeh, N., Ranjbar, R., Moradzadeh, M., Azarpira, S., Akhond, M. R., & Hashemzadeh, M. (2019). Virulence-associated genes and drug susceptibility patterns of uropathogenic Escherichia coli isolated from patients with urinary tract infection. *Infection & Drug Resistance, 12*, 2039-2047. https://doi.org/10.2147/IDR.S199764

Shi, H., Li, T., Xu, J., Yu, J., Yang, S., Zhang, X., Tao, S., Gu, J., & Deng, J. (2021). MgrB inactivation confers trimethoprim resistance in Escherichia coli. *Frontiers in Microbiology, 12*, 1-11. https://doi.org/10.3389/fmicb.2021.682205

Sierra, Y., Tubau, F., González-Díaz, A., Carrera-Salinas, A., Moleres, J., Bajanca-Lavado, P., Garmendia, J., Domínguez, M. Á., Ardanuy, C., & Martí, S. (2020). Assessment of trimethoprim-sulfamethoxazole susceptibility testing methods for fastidious Haemophilus spp. *Clinical Microbiology and Infection, 26*(7), 1-7. https://doi.org/10.1016/j.cmi.2019.11.022

Silverman, J. A., Schreiber, H. L., Hultgren, S. J., & Hooton, T. M. (2013). From physiology to pharmacy: Developments in the pathogenesis and treatment of recurrent urinary tract infections. *Current Urology Reports, 14*(5), 448-456. https://doi.org/10.1007/s11934-013-0354-5

Sköld, O. (2000). Sulfonamide resistance: Mechanisms and trends. *Drug Resistance Updates, 3*(3), 155-160. https://doi.org/10.1054/drup.2000.0146

Somorin, Y. M., Weir, N.-J. M., Pattison, S. H., Crockard, M. A., Hughes, C. M., Tunney, M. M., & Gilpin, D. F. (2022). Antimicrobial resistance in urinary pathogens and culture-independent detection of trimethoprim resistance in urine from patients with urinary tract infection. *BMC Microbiology, 22*(1), 1-8. https://doi.org/10.1186/s12866-022-02551-9

Soto, S. M., Smithson, A., Horcajada, J. P., Martinez, J. A., Mensa, J. P., & Vila, J. (2006). Implication of biofilm formation in the persistence of urinary tract infection caused by uropathogenic Escherichia coli. *Clinical Microbiology & Infection, 12*(10), 1034-1036. https://doi.org/10.1111/j.1469-0691.2006.01543.x

Sparbier, K., Schubert, S., & Kostrzewa, M. (2016). MBT-ASTRA: A suitable tool for fast antibiotic susceptibility testing? *Methods, 104*, 48-54. https://doi.org/10.1016/j.ymeth.2016.01.008

Sprouffske, K., & Wagner, A. (2016). Growthcurver: An R package for obtaining interpretable metrics from microbial growth curves. *BMC Bioinformatics, 17*(1), 1-4. https://doi.org/10.1186/s12859-016-1016-7

Stokes, H. W., & Gillings, M. R. (2011). Gene flow, mobile genetic elements and the recruitment of antibiotic resistance genes into Gram-negative pathogens. *FEMS Microbiology Reviews, 35*(5), 790-819. https://doi.org/10.1111/j.1574-6976.2011.00273.x

Su, M., Satola, S. W., & Read, T. D. (2019). Genome-based prediction of bacterial antibiotic resistance. *Journal of Clinical Microbiology, 57*(3), 1-15. https://doi.org/10.1128/jcm.01405-18

Suarez, S. A., & Martiny, A. C. (2024). Intraspecific variation in antibiotic resistance potential within E. coli. *Microbiology Spectrum, 12*(6), e03162-03123. https://doi.org/10.1128/spectrum.03162-23

Subashchandrabose, S., Hazen, T. H., Brumbaugh, A. R., Himpsl, S. D., Smith, S. N., Ernst, R. D., Rasko, D. A., & Mobley, H. L. T. (2014). Host-specific induction of Escherichia coli fitness genes during human urinary tract infection. *PNAS, 111*(51), 18327-18332. https://doi.org/10.1073/pnas.1415959112

Subhadra, B., Kim, D. H., Kim, J., Woo, K., Sohn, K. M., Kim, H. J., Han, K., Oh, M. H., & Choi, C. H. (2018). Complete genome sequence of uropathogenic Escherichia coli isolate UPEC 26-1. *Genes Genomics, 40*(6), 643-655. https://doi.org/10.1007/s13258-018-0665-5

Sultan, I., Rahman, S., Jan, A. T., Siddiqui, M. T., Mondal, A. H., & Haq, Q. M. R. (2018). Antibiotics, resistome and resistance mechanisms: A bacterial perspective. *Frontiers in Microbiology, 9*, 1-16. https://doi.org/10.3389/fmicb.2018.02066

Sun, J., Lu, F., Luo, Y., Bie, L., Xu, L., & Wang, Y. (2023). OrthoVenn3: an integrated platform for exploring and visualizing orthologous data across genomes. *Nucleic Acids Research, 51*(W1), W397-W403. https://doi.org/10.1093/nar/gkad313

Sundström, L., Rådström, P., Swedberg, G., & Sköld, O. (1988). Site-specific recombination promotes linkage between trimethoprim- and sulfonamide resistance genes. Sequence characterization of dhfrV and sulI and a recombination active locus of Tn21. *Molecular and General Genetics MGG, 213*(2), 191-201. https://doi.org/10.1007/BF00339581

Sundström, L., Swedberg, G., & Sköld, O. (1993). Characterization of transposon Tn5086, carrying the site-specifically inserted gene dhfrVII mediating trimethoprim resistance. *Journal of Bacteriology, 175*(6), 1796-1805. https://doi.org/10.1128/jb.175.6.1796-1805.1993

Sung, K., Nawaz, M., Park, M., Chon, J., Khan, S. A., Alotaibi, K., & Khan, A. A. (2024). Comprehensive Genomic Analysis of Uropathogenic E. coli: Virulence Factors, Antimicrobial Resistance, and Mobile Genetic Elements. *Pathogens, 13*(9), 1-22. https://doi.org/10.3390/pathogens13090794

Tamer, Y. T., Gaszek, I. K., Reynolds, K. A., Toprak, E., Abdizadeh, H., Atilgan, A. R., Atilgan, C., & Batur, T. A. (2019). High-order epistasis in catalytic power of dihydrofolate reductase gives rise to a rugged fitness landscape in the presence of trimethoprim selection. *Molecular Biology and Evolution, 36*(7), 1533-1550. https://doi.org/10.1093/molbev/msz086

Tamura, K., Stecher, G., & Kumar, S. (2021). MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Mol Biol Evol, 38*(7), 3022-3027. https://doi.org/10.1093/molbev/msab120

Tantoso, E., Eisenhaber, B., Kirsch, M., Shitov, V., Zhao, Z., & Eisenhaber, F. (2022). To kill or to be killed: pangenome analysis of Escherichia coli strains reveals a tailocin specific for pandemic ST131. *BMC Biology, 20*(1), 146. https://doi.org/10.1186/s12915-022-01347-7

Teelucksingh, T., Thompson, L. K., & Cox, G. (2020). The Evolutionary Conservation of Escherichia coli Drug Efflux Pumps Supports Physiological Functions. *Journal of Bacteriology, 202*(22), 1-17. https://doi.org/10.1128/jb.00367-20

Terlizzi, M. E., Gribaudo, G., & Maffei, M. E. (2017). UroPathogenic Escherichia coli (UPEC) infections: Virulence factors, bladder responses, antibiotic, and non-antibiotic antimicrobial strategies. *Frontiers in Microbiology, 8*, 1-23. https://doi.org/10.3389/fmicb.2017.01566

Thänert, R., Wang, B., Schwartz, D. J., Dantas, G., Burnham, C. A. D., Reske, K. A., Hink, T., Wallace, M. A., Seiler, S., Cass, C., Dubberke, E. R., & Kwon, J. H. (2019). Comparative genomics of antibiotic-resistant uropathogens implicates three routes for recurrence of urinary tract infections. *mBio, 10*(4). https://doi.org/10.1128/mBio.01977-19

Then, R. L. (1982). Mechanisms of resistance to trimethoprim, the sulfonamides, and trimethoprim-sulfamethoxazole. *Reviews of Infectious Diseases, 4*(2), 261-269. [Accessed on 05 September 2023] from https://www.jstor.org/stable/4452717

Thomas, M., Duffy, E., Tomlin, A., & Tilyard, M. (2020). Reduced community antibiotic dispensing in New Zealand during 2015-2018: Marked variation in relation to primary health organisation. *133*(1518), 33-42. [Accessed on 11 October 2023] from https://journal.nzma.org.nz/journal-articles/reduced-community-antibiotic-dispensing-in-new-zealand-during-2015-2018-marked-variation-in-relation-to-primary-health-organisation

Thota, S. S., & Chubiz, L. M. (2019). Multidrug Resistance Regulators MarA, SoxS, Rob, and RamA Repress Flagellar Gene Expression and Motility in Salmonella enterica Serovar Typhimurium. *Journal of Bacteriology, 201*(23), 1-18. https://doi.org/10.1128/jb.00385-19

Toro, M. d., Garcilláon-Barcia, M. P., & Cruz, F. D. L. (2014). Plasmid Diversity and Adaptation Analyzed by Massive Sequencing of Escherichia coli Plasmids. *Microbiology Spectrum, 2*(6), 1-16. https://doi.org/10.1128/microbiolspec.plas-0031-2014

Totsika, M., Gomes Moriel, D., Idris, A., Rogers, B., Wurpel, D., Phan, D., Paterson, D., & Schembri, M. (2012). Uropathogenic Escherichia coli Mediated Urinary Tract Infection. *Current drug targets, 13*, 1386-1399. https://doi.org/10.2174/138945012803530206

Touchon, M., Moura de Sousa, J. A., & Rocha, E. P. C. (2017). Embracing the enemy: the diversification of microbial gene repertoires by phage-mediated horizontal gene transfer. *Current Opinion in Microbiology, 38*, 66-73. https://doi.org/10.1016/j.mib.2017.04.010

Touchon, M., Perrin, A., de Sousa, J. A. M., Vangchhia, B., Burn, S., O'Brien, C. L., Denamur, E., Gordon, D., & Rocha, E. P. C. (2020). Phylogenetic background and habitat drive the genetic diversification of Escherichia coli. *PLOS Genetics, 16*(6), e1008866. https://doi.org/10.1371/journal.pgen.1008866

Toulouse, J. L., Lemay-St-Denis, C., Pelletier, J. N., Shi, G., Ji, X., Ebert, M. C. C. J. C., Deon, D., Gagnon, M., Ruediger, E., Marinier, A., Saint-Jacques, K., Forge, D., & Vanden Eynde, J. J. (2020). Dual-target inhibitors of the folate pathway inhibit intrinsically trimethoprim-resistant DfrB dihydrofolate reductases. *ACS Medicinal Chemistry Letters, 11*(11), 2261-2267. https://doi.org/10.1021/acsmedchemlett.0c00393

Turnidge, J., Kahlmeter, G., & Kronvall, G. (2006). Statistical characterisation of bacterial wild-type MIC value distributions and the determination of epidemiological cut-off values. *Clinical Microbiology and Infection, 12*(5), 418-425. https://doi.org/10.1111/j.1469-0691.2006.01377.x

Tuttle, A. R., Trahan, N. D., & Son, M. S. (2021). Growth and Maintenance of Escherichia coli Laboratory Strains. *Current Protocols, 1*(1), e20. https://doi.org/10.1002/cpz1.20

Tyson, G. H., McDermott, P. F., Li, C., Chen, Y., Tadesse, D. A., Mukherjee, S., Bodeis-Jones, S., Kabera, C., Gaines, S. A., Loneragan, G. H., Edrington, T. S., Torrence, M., Harhay, D. M., & Zhao, S. (2015). WGS accurately predicts antimicrobial resistance in Escherichia coli. *Journal of Antimicrobial Chemotherapy, 70*(10), 2763-2769. https://doi.org/10.1093/jac/dkv186

Uddin, T. M., Chakraborty, A. J., Khusro, A., Zidan, B. M. R. M., Mitra, S., Emran, T. B., Dhama, K., Ripon, M. K. H., Gajdács, M., Sahibzada, M. U. K., Hossain, M. J., & Koirala, N. (2021). Antibiotic resistance in microbes: History, mechanisms, therapeutic strategies and future prospects. *Journal of Infection and Public Health, 14*(12), 1750-1766. https://doi.org/10.1016/j.jiph.2021.10.020

Uhlich, G. A., Andreozzi, E., Cottrell, B. J., Reichenberger, E. R., Zhang, X., & Paoli, G. C. (2018). Sulfamethoxazole – Trimethoprim represses csgD but maintains virulence genes at 30°C in a clinical Escherichia coli O157:H7 isolate. *PLoS ONE, 13*(5), 1-22. https://doi.org/10.1371/journal.pone.0196271

Ukah, U. V., Glass, M., Avery, B., Daignault, D., Mulvey, M. R., Reid-Smith, R. J., Parmley, E. J., Portt, A., Boerlin, P., & Manges, A. R. (2018). Risk factors for acquisition of multidrug-resistant Escherichia coli and development of community-acquired urinary tract infections. *Epidemiology & Infection, 146*(1), 46-57. https://doi.org/10.1017/S0950268817002680

Ussher, J. E., McAuliffe, G. N., Elvy, J. A., & Upton, A. (2020). Appropriateness of trimethoprim as empiric treatment for cystitis in 15-55 year-old women: An audit *The New Zealand Medical Journal, 133*(1519), 62-69. [Accessed on 04 April 2023] from https://pubmed.ncbi.nlm.nih.gov/32777796/#:~:text=Conclusion%3A%20Similar%20rates%20of%20resistance,for%20cystitis%20in%20this%20group.

Valiatti, T. B., Santos, F. F., Santos, A. C. M., Nascimento, J. A. S., Silva, R. M., Gomes, T. A. T., Carvalho, E., & Sinigaglia, R. (2020). Genetic and virulence characteristics of a hybrid atypical enteropathogenic and uropathogenic Escherichia coli (aEPEC/UPEC) strain. *Frontiers in Cellular and Infection Microbiology, 10*, 1-17. https://doi.org/10.3389/fcimb.2020.00492

van Belkum, A., Burnham, C.-A. D., Rossen, J. W. A., Mallard, F., Rochas, O., & Dunne, W. M. (2020). Innovative and rapid antimicrobial susceptibility testing systems. *Nature Reviews Microbiology, 18*(5), 299-311. https://doi.org/10.1038/s41579-020-0327-x

van Hecke, O., Wang, K., Lee, J. J., Roberts, N. W., & Butler, C. C. (2017). Implications of Antibiotic Resistance for Patients' Recovery From Common Infections in the Community: A Systematic Review and Meta-analysis. *Clinical Infectious Diseases, 65*(3), 371-382. https://doi.org/10.1093/cid/cix233

Versluis, A., Schnoor, K., Chavannes, N. H., & Talboom-Kamp, E. P. W. A. (2022). Direct access for patients to diagnostic testing and results using eHealth: Systematic review on eHealth and diagnostics. *Journal of Medical Internet Research, 24*(1), 1-21. https://doi.org/10.2196/29303

Vinchhi, R., Jena, C., & Matange, N. (2023). Adaptive laboratory evolution of antimicrobial resistance in bacteria for genetic and phenotypic analyses. *STAR Protocols, 4*(1). https://doi.org/10.1016/j.xpro.2022.102005

Viveiros, M., Dupont, M., Rodrigues, L., Couto, I., Davin-Regli, A., Martins, M., Pagès, J.-M., & Amaral, L. (2007). Antibiotic Stress, Genetic Response and Altered Permeability of E. coli. *PLoS ONE, 2*(4), e365. https://doi.org/10.1371/journal.pone.0000365

Wagenlehner, F. M. E., Bjerklund Johansen, T. E., Cai, T., Koves, B., Kranz, J., Pilatz, A., & Tandogdu, Z. (2020). Epidemiology, definition and treatment of complicated urinary tract infections. *Nature Reviews Urology, 17*(10), 586-600. https://doi.org/10.1038/s41585-020-0362-4

Wald-Dickler, N., Holtom, P., & Spellberg, B. (2017). Busting the Myth of "Static vs Cidal": A Systemic Literature Review. *Clinical Infectious Diseases, 66*(9), 1470-1474. https://doi.org/10.1093/cid/cix1127

Wald-Dickler, N., Holtom, P., & Spellberg, B. (2018). Busting the myth of "static vs cidal": A systemic literature review. *Clinical Infectious Diseases, 66*(9), 1470-1474. https://doi.org/10.1093/cid/cix1127

Walsh, T. R., Gales, A. C., Laxminarayan, R., & Dodd, P. C. (2023). Antimicrobial Resistance: Addressing a Global Threat to Humanity. *PLoS Medicine, 20*(7), e1004264. https://doi.org/10.1371/journal.pmed.1004264

Wanda, C. R. (2018). An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS Microbiology, 4*(3), 482-501. https://doi.org/10.3934/microbiol.2018.3.482

Wang, F., Zhou, H., Olademehin, O. P., Kim, S. J., & Tao, P. (2018). Insights into key interactions between vancomycin and bacterial cell wall structures. *ACS Omega, 3*(1), 37-45. https://doi.org/10.1021/acsomega.7b01483

Wang, Y., Zhao, Y., Bollas, A., Wang, Y., & Au, K. F. (2021). Nanopore sequencing technology, bioinformatics and applications. *Nature Biotechnology 39*(11), 1348-1365. https://doi.org/10.1038/s41587-021-01108-x

Wang, Z., Gerstein, M., & Snyder, M. (2009). RNA-Seq: A revolutionary tool for transcriptomics. *Nature Reviews Genetics 10*(1), 57-63. https://doi.org/10.1038/nrg2484

Warren, J. W., Diggs, C., Horne, L., & Greenberg, P. (2011). Interstitial cystitis/painful bladder syndrome: What do patients mean by "perceived" bladder pain? *Urology, 77*(2), 309-312. https://doi.org/10.1016/j.urology.2010.08.030

Wendling, C. C., Refardt, D., & Hall, A. R. (2021). Fitness benefits to bacteria of carrying prophages and prophage-encoded antibiotic-resistance genes peak in different environments. *Evolution, 75*(2), 515-528. https://doi.org/10.1111/evo.14153

Wenzler, E., Maximos, M., Asempa, T. E., Biehle, L., Schuetz, A. N., & Hirsch, E. B. (2023). Antimicrobial susceptibility testing: An updated primer for clinicians in the era of antimicrobial resistance: Insights from the Society of Infectious Diseases Pharmacists. *Pharmacotherapy, 43*(4), 264-278. https://doi.org/10.1002/phar.2781

Whelan, S., Lucey, B., & Finn, K. (2023). Uropathogenic Escherichia coli (UPEC)-Associated Urinary Tract Infections: The Molecular Basis for Challenges to Effective Treatment. *Microorganisms, 11*(9), 1-32. https://doi.org/10.3390/microorganisms11092169

White, P. A., McIver, C. J., Deng, Y.-M., & Rawlinson, W. D. (2000). Characterisation of two new gene cassettes, aadA5 and dfrA17. *FEMS Microbiology Letters, 182*(2), 265-269. https://doi.org/10.1111/j.1574-6968.2000.tb08906.x

Wick, R. R., Judd, L. M., Gorrie, C. L., & Holt, K. E. (2017). Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput Biol, 13*(6), 1-22. https://doi.org/10.1371/journal.pcbi.1005595

Wick, R. R., Schultz, M. B., Zobel, J., & Holt, K. E. (2015). Bandage: interactive visualization of de novo genome assemblies. *Bioinformatics, 31*(20), 3350-3352. https://doi.org/10.1093/bioinformatics/btv383

Wiegand, I., Hilpert, K., & Hancock, R. E. W. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature Protocols, 3*(2), 163-175. https://doi.org/10.1038/nprot.2007.521

Williamson, D. A., & Heffernan, H. (2014). The changing landscape of antimicrobial resistance in New Zealand. *New Zealand Medical Journal* 41-54. [Accessed on 05 September 2023] from https://pubmed.ncbi.nlm.nih.gov/25290498/

Williamson, D. A., Roos, R., Verrall, A., Smith, A., & Thomas, M. G. (2016). Trends, demographics and disparities in outpatient antibiotic consumption in New Zealand: A national study. *Journal of Antimicrobial Chemotherapy, 71*(12), 3593-3598. https://doi.org/10.1093/jac/dkw345

Witzany, C., Rolff, J., Regoes, R. R., & Igler, C. (2023). The pharmacokinetic–pharmacodynamic modelling framework as a tool to predict drug resistance evolution. *Microbiology, 169*(7). https://doi.org/10.1099/mic.0.001368

World Health Organisation. (2024). *WHO list of medically important antimicrobials: A risk management tool for mitigating antimicrobial resistance due to non-human use*. https://cdn.who.int/media/docs/default-source/gcp/who-mia-list-2024-lv.pdf

Wrobel, A., Arciszewska, K., Maliszewski, D., & Drozdowska, D. (2020). Trimethoprim and other nonclassical antifolates an excellent template for searching modifications of dihydrofolate reductase enzyme inhibitors. *Journal of Antibiotics, 73*(1), 5-27. https://doi.org/10.1038/s41429-019-0240-6

Wu, K.-Y., Cao, B., Wang, C.-X., Yang, X.-L., Zhao, S.-J., Diao, T.-Y., Lin, L.-R., Zhao, G.-X., Zhou, W., Yang, J.-R., & Li, K. (2022). The C5a/C5aR1 axis contributes to the pathogenesis of acute cystitis through enhancement of adhesion and colonization of uropathogenic E. coli. *Frontiers in Cellular & Infection Microbiology, 12*, 1-15. https://doi.org/10.3389/fcimb.2022.824505

Wüthrich, D., Brilhante, M., Hausherr, A., Becker, J., Meylan, M., & Perreten, V. (2019). A novel trimethoprim resistance gene, dfrA35, characterized from Escherichia coli from calves. *mSphere, 4*(3), 1-7. https://doi.org/10.1128/mSphere.00255-19

Xin, Y., Xie, J., Nan, B., Tang, C., Xiao, Y., Wu, Q., Lin, Y., Zhang, X., & Shen, H. (2021). Freeze-Thaw Pretreatment Can Improve Efficiency of Bacterial DNA Extraction From Meconium. *Frontiers in Microbiology, 12*. https://doi.org/10.3389/fmicb.2021.753688

Yamamoto, S., Tsukamoto, T., Terai, A., Kurazono, H., Takeda, Y., & Yoshida, O. (1997). Genetic evidence supporting the fecal-perineal-urethral hypothesis in cystitis caused by Escherichia coli. *The Journal of Urology, 157*(3), 1127-1129. https://doi.org/10.1016/S0022-5347(01)65154-1

Yang, D., Jiang, F., Huang, X., Li, G., & Cai, W. (2021). Transcriptomic and metabolomic profiling reveals that KguR broadly impacts the physiology of uropathogenic Escherichia coli under in vivo relevant conditions. *Frontiers in Microbiology, 12*, 1-12. https://doi.org/10.3389/fmicb.2021.793391

Yang, J., Qiu, X., Zhou, M., & Wang, D. (2021). Crocetin attenuating urinary tract infection and adherence of uropathogenic E. coli in NRK-52E cells via an inflammatory pathway. *Journal of Food Biochemistry, 45*(12), 1-12. https://doi.org/10.1111/jfbc.13998

Ye, C., Yang, Y., Yang, L., Zeng, X., Qiao, S., Chen, X., Hua, X., & Yang, M. (2022). Metabolic engineering of Escherichia coli BW25113 for the production of 5-Aminolevulinic Acid based on CRISPR/Cas9 mediated gene knockout and metabolic pathway modification. *Journal of Biological Engineering, 16*(1). https://doi.org/10.1186/s13036-022-00307-7

Young, H.-K., Qumsieh, M. J., & Mclntosh, M. L. (1994). Nucleotide sequence and genetic analysis of the type Ib trimethoprim-resistant, Tn4132-encoded dihydrofolate reductase. *Journal of Antimicrobial Chemotherapy, 34*(5), 715-725. https://doi.org/10.1093/jac/34.5.715

Yu, H. S., Lee, J. C., Kang, H. Y., Jeong, Y. S., Lee, E. Y., Choi, C. H., Tae, S. H., Lee, Y. C., Seol, S. Y., & Cho, D. T. (2004). Prevalence of dfr genes associated with integrons and dissemination of dfrA17 among urinary isolates of Escherichia coli in Korea. *Journal of Antimicrobial Chemotherapy, 53*(3), 445-450. https://doi.org/10.1093/jac/dkh097

Zagaglia, C., Maurizi, L., Longhi, C., Ammendolia, M. G., & Nicoletti, M. (2022). Urinary tract infections caused by uropathogenic Escherichia coli strains—new strategies for an old pathogen. *Microorganisms, 10*(7). https://doi.org/10.3390/microorganisms10071425

Zampieri, M., Enke, T., Chubukov, V., Ricci, V., Piddock, L., & Sauer, U. (2017). Metabolic constraints on the evolution of antibiotic resistance. *Molecular Systems Biology, 13*(3), 917. https://doi.org/10.15252/msb.20167028

Zeng, Q., Xiao, S., Gu, F., He, W., Xie, Q., Yu, F., & Han, L. (2021). Antimicrobial resistance and molecular epidemiology of uropathogenic Escherichia coli isolated from female patients in Shanghai, China. *Frontiers in Cellular & Infection Microbiology, 11*, 1-12. https://doi.org/10.3389/fcimb.2021.653983

Zhanel, G. G., Hisanaga, T. L., Laing, N. M., DeCorby, M. R., Karlowsky, J. A., Hoban, D. J., Nichol, K. A., Weshnoweski, B., Johnson, J., Noreddin, A., & Low, D. E. (2006). Antibiotic resistance in Escherichia coli outpatient urinary isolates: Final results from the North American Urinary Tract Infection Collaborative Alliance (NAUTICA). *International Journal of Antimicrobial Agents, 27*(6), 468-475. https://doi.org/10.1016/j.ijantimicag.2006.02.009

Zhang, X., Xiao, S., Jiang, X., Li, Y., Fan, Z., Yu, Y., Wang, P., Li, D., Zhao, X., & Liu, C. (2019). Genomic characterization of Escherichia coli LCT-EC001, an extremely multidrug-resistant strain with an amazing number of resistance genes. *Gut Pathogens, 11*(1), 1-8. https://doi.org/10.1186/s13099-019-0298-5

Zhao, R., & Goldman, I. D. (2003). Resistance to antifolates. *Oncogene, 22*(47), 7431-7457. https://doi.org/10.1038/sj.onc.1206946

Zhou, Y., Zhou, Z., Zheng, L., Gong, Z., Li, Y., Jin, Y., Huang, Y., & Chi, M. (2023). Urinary tract infections caused by uropathogenic Escherichia coli : Mechanisms of infection and treatment options. *International Journal of Molecular Sciences, 24*(13), 1-33. https://doi.org/10.3390/ijms241310537

Appendices

Appendix A: Middlemore Hospital Antibiotic Susceptibility Testing

Antibiotics tested during disk diffusions assays at Middlemore Hospital as mentioned in Section 2.2.1. Isolates which were classified as being TMP resistant were given to AUT for further testing.

Table A: Antibiotics used during AST patient urine testing at Middlemore Hospital.

Antimicrobial Class	Antibiotic	Disk Content
Penicillins	Ampicillin	10 µg
β-Lactam Combination Agents	Amoxicillin-Clavulanic Acid	20-10 µg
Monobactams	Aztreonam	30 µg
Cephems	Cefoxitin	30 µg
	Cefuroxime	30 µg
	Ceftazidime	30 µg
	Ceftriaxone	30 µg
Carbapenems	Ertapenem	10 µg
Aminoglycosides	Gentamicin	10 µg
Nitrofurans	Nitrofurantoin	300 µg
Quinolones	Norfloxacin*	10 µg
Folate Pathway Antagonists	Trimethoprim	5 µg

Cephazolin (30 µg) used in place of norfloxacin in paediatrics (≤ 14 years).

Appendix B: Confirmation of Antibiotic Susceptibility

Full antibiotic susceptibility results for the 106 isolates obtained from Middlemore Hospital from Section 2.3.1 can be viewed in Supplementary File Appendix B. Duplicate zones of inhibition were calculated and susceptibility was interpreted for each antibiotic based on CLSI (2023). Green indicates isolate is susceptible, yellow indicates intermediate susceptibility, and red indicates resistant to antibiotic. NG indicates no growth of isolate and l.o.g, indicates lawn of growth up to antibiotic disk.

Appendix C: Determination of Resistance Levels

Growth curves generated for 74 isolates from Section 2.3.2.1 can be viewed in Supplementary File Appendix C. These curves were used to generate the two criteria in Table 2.5 and Table 2.6.

Appendix D: OrthoVenn3 Gene Cluster Occurrence

The OrthoVenn3 cluster occurrence table from Section 3.3.3.1 of the 3520 clusters among the six resistant isolates and the susceptible strain and can be viewed in Supplementary File Appendix D.

Appendix E: OrthoVenn3 Resistance Cluster Biological Processes

The 152 clusters of interest associated with AMR and pathogenicity between the six resistant isolates and the BLASTp and InterPro results from Section 3.3.3.1 can be found in in Supplementary File Appendix E**.**

Appendix F: OrthoVenn3 Resistance

The OrthoVenn3 identification of the 152 biological processes clusters shared between the six resistance isolates from Section 3.3.3.1 can be viewed in Supplementary File Appendix F.