

The assessment of antimicrobial resistance from three perspectives: disinfection, surveillance and drug development.

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Abstract

The increase of antimicrobial resistance (AMR) is a major threat to public health. Overuse of antibiotics, combined with the diminishment of the antibiotic pipeline and genetic plasticity of microbes, places humanity in a compromised position in the fight against pathogenic bacteria. A wide range of preventative measures must be implemented to slow down the spread of resistance and avoid a post-antibiotic era, where even common infections become challenging to treat. While the urgent need to tackle the AMR crisis has been widely acknowledged, its complexity combined with the dramatic adaptability of bacteria has proven challenging. A multidisciplinary, cohesive approach is necessary to effectively find solutions to the AMR problem. The following project addressed the issue of AMR from three different perspectives. Firstly, we have assessed the effectiveness of current alcohol disinfection methods, used commonly in the clinic, and reported an alarming increase in the isopropanol tolerance of *Enterococcus faecium* bacterial strains. This was followed by an AMR surveillance report, assessing the spread of extended spectrum β -lactamase *Escherichia coli* isolates, in North Carolina. Lastly, we have analysed the potential use of polymeric nanoparticles as antibacterial agents, showing their diverse applications in bacteria eradication. By focusing on the above issues, the following project addresses the AMR challenge from three important perspectives, namely; disinfection of clinical settings, AMR surveillance and new drug development. This multidisciplinary approach demonstrates the importance of a cohesive methodology, when tackling AMR infections, demonstrating that the cooperation of different research fields is necessary to fully understand the complexity of AMR.

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Table of abbreviations

AMR	antimicrobial resistance
CDC	Centres for disease control and prevention
ESBL	extended spectrum β -lactamases
FQ	fluoroquinolones
HGT	horizontal gene transfer
MDR	multi-drug resistant
MDRE	multidrug resistant enterococci
NP	nanoparticle
PI	propidium iodide
PNP	polymeric nanoparticle
SYBRG	SYBR Green I
QS	quorum sensing
WHO	World Health Organization

1. Introduction

1.1 The role of antibiotics

The discovery of antibiotics has enabled the control of infectious diseases, once leading causes of human morbidity and mortality [1]. It is not an understatement to say, that since their introduction in the 1940s, antibiotics have become one of the most successful and vital chemical treatments in medicine. The antibiotic era we live in today, arose from the discoveries of Paul Ehrlich and Alexander Fleming. Ehrlich's idea of a "magic bullet" capable of selectively targeting pathogens within the human body led to the development of Salvarsan, an arsenic based chemical used to treat syphilis [2]. While the drug was not an antibiotic in the strict sense of the word, Ehrlich's systematic screening approach became the cornerstone of drug discovery strategies applied in the pharmaceutical industry [1]. Flemings discovery of penicillin in 1928 was preceded by the use of mould to inhibit bacterial growth since ancient times. Fleming realized the great potential of penicillin and persistently aimed to overcome the significant challenges associated with its purification. His research, combined with the discovery of an effective purification technique by Florey and Chain, led to penicillin becoming available in 1945 [2]. Interestingly it was also Fleming who warned of resistance to penicillin developing, if used in insufficient amounts [1].

The discovery of penicillin was followed by a golden age of antibiotic development, between the 1940s and the 1970s, when most of the current treatments were discovered (**Table 1.1**). However, despite recent commercialisation of synthetic derivatives of ketolides and quinolones, most classes of antibiotics currently in use originate from the 1980s, showing a significant stagnation within the sector [3]. Due to the lack of return on investment and emerging resistance, the pharmaceutical industry has gradually abandoned antibiotic research, in favour of investing in the more profitable chronic diseases [3]. Of the 20 pharmaceutical companies that invested in antibiotic discovery in the 1980s, only 5 were left by 2015 [4]. This drastically diminished antibiotic pipeline, combined with increasing antibiotic resistance present within bacteria populations, could have dramatic consequences in the near future [5].

Table 1.1: Summary of the main antibiotic classes with drug examples and discovery dates.

Antibiotic class	Mode of action	Example drug	Drug discovery date
β -lactams	Inhibition of cell wall synthesis	Penicillin	1928 [2]
		Cephalosporins	1948 [5]
		Carbapenems	1976 [5]
		Monobactams	1981 [5]
Aminoglycosides	Inhibition of protein synthesis	Streptomycin	1943 [6]
Glycopeptides	Inhibition of cell wall synthesis	Vancomycin	1953 [7]
Ketolides	Inhibition of protein synthesis	Telithromycin	1997 [5]
Lincosamides	Inhibition of protein synthesis	Lincomycin	1962 [8]
Lipopeptides	Inhibition of cell wall synthesis	Daptomycin	1986 [3]
Macrolides	Inhibition of protein synthesis	Erythromycin	1952 [9]
Oxazolidinones	Inhibition of protein synthesis	Linezolid	1987 [3]
Polypeptides	Membrane disorganizing agents	Polymyxin	1947 [10]
Quinolones	Inhibition of DNA synthesis	Nalidixic acid	1960 [11]
Rifamycins	Inhibition of RNA synthesis	Rifampicin	1957 [12]
Sulfonamides	Competitive inhibition of folic acid synthesis	Trimethoprim/sulfamethoxazole	1968 [13]
Tetracyclines	Inhibition of protein synthesis	Chlortetracycline	1948 [14]
Streptogramins	Inhibition of protein synthesis	Streptogramin B	1953 [15]
Tuberculosis drugs	Inhibition of cell wall synthesis	Ethambutol	1961 [16]

1.2 Antibiotic resistance

Bacterial resistance has been a recognized risk, since the beginning of the antibiotic era. The natural environment harbours a diverse library of resistance determinants, some dating back millions of years and some arising suddenly from mutations [17]. Since the introduction of penicillin in the 1940s antibiotic resistance genes have been introduced world-wide at a

dramatic pace. The overuse of antibiotics both in the clinic and in the agricultural and livestock industries has led to a significant increase in AMR (**Figure 1.1**). Of particular concern is the much higher use of agricultural antibiotics, compared to their amounts prescribed for humans, including the use of “last-resort” treatments. Within the US more than 70% of medically important antibiotics are used in livestock, compared to only 30% in humans [18]. This dramatic disproportion has a significant impact on the increase of antibiotic resistance. Antibiotics of pharmaceutical origin can now be found in large quantities in man-made environments, such as sewage and waste water treatment plants, forming a rich reservoir of resistance genes transferred further into terrestrial, freshwater and marine environments [19].

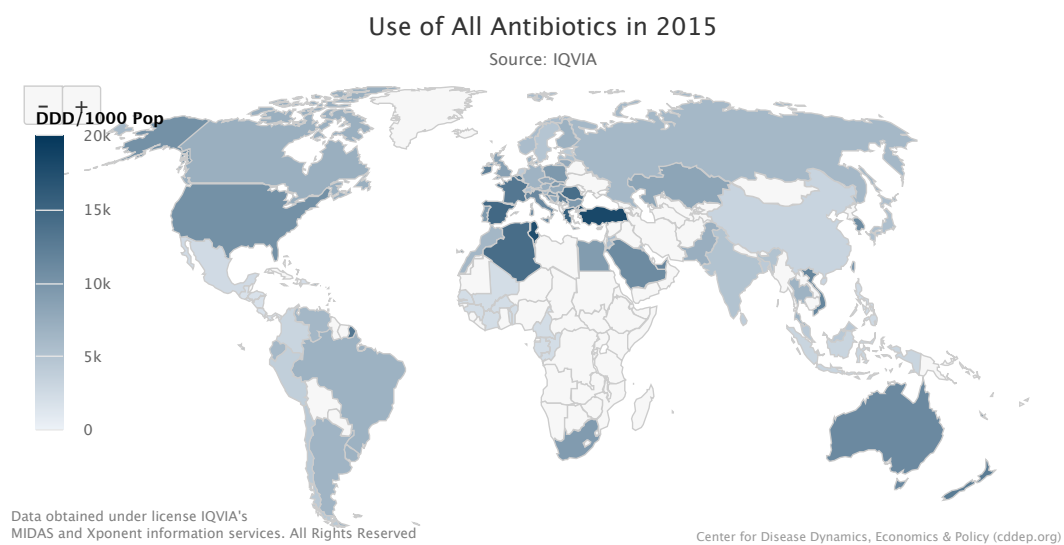


Figure 1.1: The worldwide use of antibiotics in 2015. Image taken from [20].

According to the 2019 report by the Centres for Disease Control and Prevention more than 2.8 million antibiotic resistant infections occur in the United States each year [21]. This increase is combined with a significant surge in healthcare costs by billions of pounds each year [22]. Furthermore, the rise in resistance does not correlate with a better understanding and regulation of antibiotic dosing. It is estimated that within the US around 47 million antibiotic treatments are prescribed each year for non-bacterial infections, making it 30% of all antibiotic prescriptions [21]. This lack of understanding of antibiotic action is also visible within the population, with only 36% of people correctly answering that antibiotics do not kill viruses in a survey conducted within the EU [5].

1.3 Resistance acquisition

The genetic plasticity of bacteria allows them to quickly respond to a range of environmental threats, including antibiotics. The overuse of antimicrobials in recent years has accelerated the pace at which bacteria acquire resistance, resulting in limited treatment options for multiple infections. From an evolutionary perspective, bacteria have developed two major genetic strategies to adapt to antibiotic presence, which involve either genetic mutations or acquisition of foreign DNA [23]. In the genetic mutation scenario, a bacterial cell, derived from a susceptible population, develops mutations in genes affecting antibiotic activity. Such transformations within the bacteria are often costly to cell homeostasis and are only maintained if necessary for cell survival. The resistant mutant is then capable of surviving the antibiotic attack and becomes the predominant genotype within the population [23]. Bacterial acquisition of foreign DNA through horizontal gene transfer (HGT) is the best-known example of rapid bacterial adaptation to environmental pressure [24]. Since the majority of antibiotics currently in use originate from natural compounds produced by soil fungi, resistance to them has always been present within the environment and can be easily transferred to other cells. Furthermore, HGT enables gene transfer within the genus level and even distantly related lineages belonging to different domains [25]. The genetic material can be transmitted through three main pathways: transformation, conjugation and transduction (phage mediated) [26]. Transformation, involving the incorporation of naked fragments of extracellular DNA, is the least common, with only a handful of clinically relevant bacteria capable of it [23]. Conjugation is based on a cell-to-cell DNA transfer using mobile genetic elements, such as plasmids. Once a resistance gene becomes established on successful plasmids it can be easily transferred between cells, as seen for the *bla*_{CTX-M} genes responsible for ESBL presence [26]. Lastly, transduction incorporates bacteriophages to carry DNA fragments ranging from chromosomal DNA to plasmids and transposons. Bacteriophages are capable of surviving in the more adverse extracellular conditions, making them perfect vehicles when transferring resistance between different biomes [27]. Once resistant, bacteria have been shown to evade antibiotic action through four main molecular mechanisms; modification of the target site, modification or destruction of the antibiotic, antibiotic efflux *via* efflux transporters and reduced antibiotic influx through decreased membrane permeability. Resistance to an antibiotic class can often originate from several of these mechanisms [28].

1.3.1 Target site modification

A key feature of antimicrobial therapies is their targeting of cellular components absent from mammalian cells. An example of such a structure is the bacterial peptidoglycan cell wall. Many of the current antimicrobial therapies, such as β -lactams, target the enzymes necessary to synthesise and assemble the peptidoglycan layer, however resistance is frequently reported [28]. Furthermore, bacterial cellular components that provide a similar function to their mammalian counterparts, but differ from them structurally, can be targeted. Such is the case for protein synthesising components, RNA polymerase, chromosome segregation and folic acid metabolism [29]. Resistance to these therapies is however highly common. A clinically problematic example is plasmid-mediated fluoroquinolone (FQ) resistance, which involves the *qnr* determinant encoding for a protein capable of protecting the targets of the antibiotic: SNA gyrase and topoisomerase IV. To date five *qnr* families have been reported, with their number likely to increase [28].

1.3.2 Antibiotic modification or destruction

Antibiotics can be destroyed through a range of pathways. These are often based on the hydrolysis of susceptible bonds, including esters and amides. A common example of such a pathway is the use of β -lactamases to cleave the amide fragment of β -lactam rings within penicillins and cephalosporins [30]. Antibiotic structure modifications originate from functional group transfers, modifying antibiotic structure and activity. An example of such a process can be seen for aminoglycoside antibiotics, which have been shown to become deactivated by acetyltransferases encoded by *aac* genes [28].

1.3.3 Efflux transporters

Bacterial cells employ efflux pumps to eliminate toxic compounds. Since the introduction of antibiotics these pumps have been incorporated to eliminate the drug molecules. Inappropriate antibiotic dosing, resulting in too low drug concentrations, has enabled bacteria to survive and adapt their efflux pumps to deal with the new danger. In many clinical isolates of antibiotic resistant gram-negative and gram-positive bacteria an upregulation in efflux transporters has been observed [31]. A classic example of efflux pump-based resistance can be seen for tetracycline antibiotics. The resistance mechanism originates from the *tet* gene, enabling the production of the Tet protein, capable of ejecting the antibiotic out of the prokaryotic cell [32].

1.3.4 Decreased membrane permeability

Many antibiotics must penetrate into the bacterial cell in order to be active. Bacteria prevent this process through a decrease in cell permeability. This is especially the case for gram-negative bacteria. The double membrane present in gram-negative cells enables an enhanced control of molecule influx through the use of water-filled porins [33]. These channels are the main delivery route for major current treatments, such as β -lactams and FQs. Bacteria with an inherently reduced outer-membrane permeability, such as *Pseudomonas aeruginosa* or *Acinetobacter baumannii* have been shown to possess an innate low susceptibility to β -lactams [34]. Porins present in gram-negative bacteria differ based on their activity, functional structure, regulation and expression. Clinical studies of antibiotic resistant strains have shown their porin profiles have been altered through either porin exchange, a decrease in porin expression or mutations in porin structure, leading to antibiotic tolerance [33].

1.4 Tackling resistance

In a report from 2017 the WHO specified a list of bacteria for which new antibiotics are urgently needed. Multi-drug resistant *Mycobacterium tuberculosis*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and ESBL-producing and carbapenem-resistant *Enterobacteriaceae* were classified as of critical priority. New treatment for resistant strains of *Enterococcus faecium*, *Staphylococcus aureus*, *Helicobacter pylori*, *Campylobacter* spp, *Salmonellae* and *Neisseria gonorrhoeae* were of high priority, while treatments for *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Shigella* spp were of medium urgency [35]. It is vital these bacteria are closely monitored and are the principal target when developing new antimicrobial therapies.

A range of approaches must be implemented to slow down the spread of resistance. Of principal importance is the enactment of antibiotic stewardship programmes, aimed at minimising unnecessary antibiotic prescriptions, optimizing clinical outcomes and reducing resistance spreading and healthcare costs [36]. Effective antibiotic stewardship programmes should be combined with comprehensive infection control policies; entailing appropriate disinfection of clinical settings, use of protective barriers such as gloves and ensuring the personal hygiene of healthcare workers [37]. Such precautions have been shown to significantly decrease the

transmission of MDR bacterial strains between patients [38]. Global surveillance of antibiotic resistance patterns is necessary, both to provide early warnings of emerging threats and to allow healthcare workers to make informed clinical decisions [39]. Control and prevention of resistance spreading must occur in parallel with the development of novel antibacterial agents. The dramatically diminished antibiotic pipeline must be replenished with new drugs, capable of effectively eradicating bacteria. To minimise the risk of resistance developing, therapies targeting pathways not essential for bacterial survival are of particular interest, due to the reduced evolutionary pressure exerted on the pathogen. These therapies include the use of quorum sensing inhibitors and nanoparticle drug carriers [40]. Lastly, it is vital that both the clinicians and the public are educated on antibiotic dosing, applications and AMR. This includes imposing strict guidelines on farmers to not use clinical antibiotics in livestock treatments [38]. All of the above strategies must be implemented together and will not be successful on their own.

1.5 Research aims

A cohesive, multidisciplinary approach to antimicrobial resistance is essential. This report addresses three aspects necessary to counter antibiotic resistance; namely hygiene and disinfection, AMR surveillance and new drug development. The following write-up addresses the AMR challenge through assessing the efficiency of alcohol sterilisation methods, currently applied in the clinic, evaluating the spread of resistant bacterial infections within community hospitals in North Carolina and looking at novel treatment methods to counter AMR, using antimicrobial nanoparticles and nanoparticle drug delivery systems. Each issue addressed is of vital importance to prevent and understand the trends in AMR dissemination.

2. Assessing *Enterococcus faecium* tolerance to isopropanol.

Supervisor: Professor Willem van Schaik, University of Birmingham

Abstract

E. faecium infections are challenging to treat due to its wide-spread resistance to most of the current treatment methods. This resistance, combined with the danger of cross-transmission to other patients, results in *E. faecium* infections becoming increasingly common in the clinical setting. Robust disinfection procedures must be applied, in order to prevent the spreading of this resilient pathogen. The introduction of alcohol disinfectants in the clinical setting has been highly successful in reducing bacterial transmission. However, there have been increasing reports of *E. faecium* displaying tolerance to alcohol-based disinfectants. These findings place the current infection control recommendations under question and must be addressed to ensure patient safety. In this study, tolerance of eight *E. faecium* strains, of diverse origin, to a range of isopropanol concentrations was assessed. First, tolerance was examined using a colony-counting method, derived from that of Miles and Misra, to assess survival after a 5-minute exposure to 23% (v/v) isopropanol. This yielded variable and non-reproducible results. In order to more accurately assess tolerance an alternative method, that measures the turbidity of cultures recovering from alcohol treatment was developed and assessed. This method proved more reproducible than the colony-counting method. A third assay based on fluorescent dye staining of alcohol-treated cultures was also developed, and preliminary results suggest that it might represent a rapid, accurate method for assessing alcohol tolerance. Results obtained from these three methods show that the majority of the analysed strains exhibit at least moderate alcohol tolerance, with high survival rates seen for isopropanol concentrations as high as 23% (v/v). Further research is required to understand the mechanisms underlying the alcohol tolerance observed here.

2.1 Introduction

Enterococci are gram-positive bacteria found in plants, soil and gastrointestinal tracts of humans, mammals and insects [41]. While most enterococcus species are commensals, aiding in digestion and metabolic pathways, some are opportunistic pathogens, posing an increasing danger to public health [42]. Within enterococci, *E. faecalis* and *E. faecium* have become particularly important causes of nosocomial infections, including endocarditis, bacteremia, neonatal infections, urinary tract, central nervous system, abdominal and pelvic infections [43]. Between 2006 - 2007 the National Healthcare Safety Network stated enterococcus species were reportedly responsible for 12% of bacteremia cases, noted within 463 hospitals, making them the second most common pathogen isolated after *S. aureus* [44].

While *E. faecalis* causes more infections, *E. faecium* has shown resistance to the widest array of antibiotics [42]. 82.2% of *E. faecium* isolates involved in healthcare-associated bloodstream infections in the United States have been reported as resistant to vancomycin, compared to 9.8% of *E. faecalis* isolates [45]. An explanation of this phenomena lies in the genetic differences between these two enterococcal groups. While no clade separation has been reported within the *E. faecalis* population, in *E. faecium* the evolution of three distinct clades: A1, A2 and B has been reported (**Figure 2.1**) [46][47]. Many of the pathways observed within clade B, found most commonly in faecal *E. faecium* isolates obtained from non-hospitalised patients, have been lost by clade A to enable rapid adaptations to environmental changes. The splitting of clade A into the multidrug resistant, clinical clade A1 and mostly animal originating clade A2 appears to have taken place after the introduction of antibiotics in clinical practice [47]. The rapidly evolving clade A1 exhibits an increase in mobile genetic elements and an alteration in metabolism and hypermutability, enabling this bacterial group to become one of the most challenging and multidrug resistant nosocomial pathogens [47].

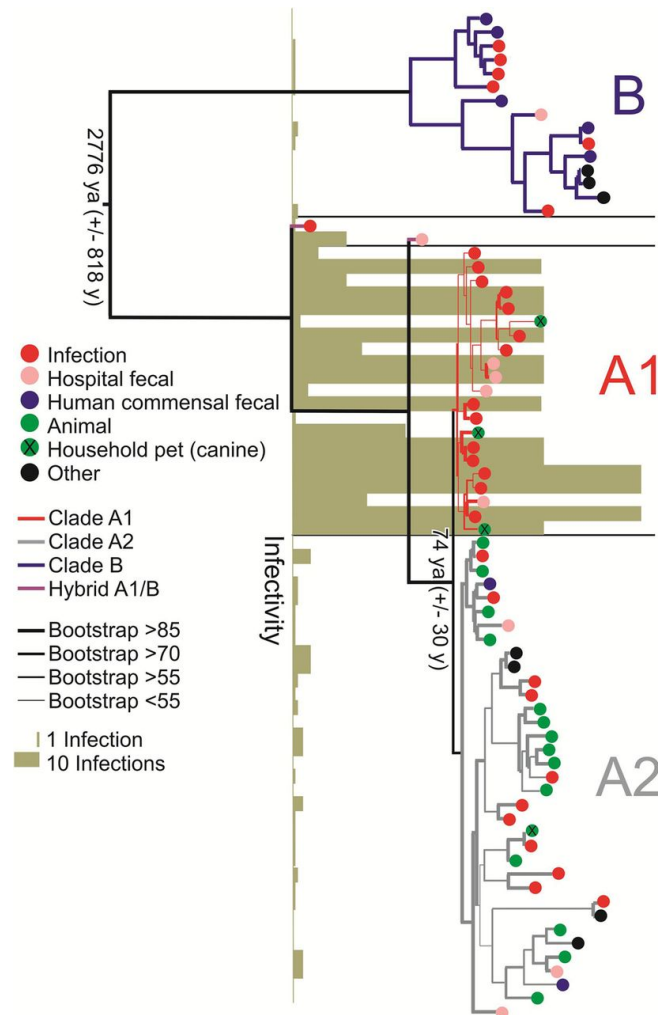


Figure 2.1: RAxML SNP-based tree showing the three distinct *E. faecium* clades. Image taken from [47].

While enterococcus virulence is lower than that of other common pathogens, severe infections can take place in vulnerable or immunocompromised patients [48]. It has been reported that approximately 30% of patients following liver transplantation are colonized with vancomycin resistant enterococci, of whom over 25% develop infection [45]. Therefore, preventative measures must be undertaken to limit the challenges associated with multidrug resistant enterococci (MDRE). Antibiotic resistant strains have been reported to transfer from contaminated hands and gloves to patients and environmental surfaces at an average rate of 10.6% [49]. Effective hand hygiene is thus a key measure to prevent and control the spread of MDRE. The WHO states hand disinfection can be performed either with plain soap or antiseptic agents including alcohols, chlorhexidine and chlorine derivatives. Alcohol-based hand rubs are especially advantageous, since they enable a fast elimination of the majority of microbes, are easily available and do not require any particular infrastructure to be used [50]. Guidelines for alcohol-based hand rubs state they should contain 70 to 90% ethanol,

isopropanol or *n*-propanol, or a combination of those products [51]. The alcohols act as bactericides by disrupting bacterial membrane functions, resulting in a loss of the cell permeability barrier [52].

The use of alcohol-based hand rubs has reportedly reduced the spread of nosocomial infections by 40% [53]. Nonetheless, a recent study by Pidot *et al.* reported an increasing resistance of *E. faecium* to 23 % (v/v) handwash isopropanol [54]. This increase in tolerance was further shown to enhance the likelihood of transmission, when subjected to a full 70% (v/v) alcohol solution. These findings potentially jeopardize the efficiency of the current sanitization methods and should be assessed further. Building on the data reported by Pidot *et al.*, our objective was the evaluation of isopropanol tolerance present in 8 *E. faecium* strains of differing origin. Previous work within the group showed isopropanol to have the strongest killing effect on *E. faecium*, when compared to ethanol and *n*-propanol. For that reason, isopropanol is the alcohol of choice for the following assays, under the assumption that strains tolerant to isopropanol will show resistance to ethanol and *n*-propanol. While the recommended isopropanol concentrations for clinical use are within the 70-90% (v/v) boundary, we have applied lower concentrations to allow a meaningful comparison between the isolates analysed. As Pidot *et al.* analysed alcohol tolerance development at the 23% (v/v) isopropanol concentration, we chose it as the upper tolerance limit throughout the majority of this study. The tested *E. faecium* strains were incubated with the selected isopropanol concentrations for 5 minutes. This incubation time has been previously applied by Pidot *et al.* and has been shown to effectively eradicate alcohol susceptible cells in previous experiments within the group [54].

2.2 Methods

2.2.1 Alcohol killing assay based on the Miles and Misra method

A protocol based on a published study was used to assess alcohol tolerance [54]. Single *E. faecium* colonies were used to inoculate 10 mL Brain Heart Infusion (BHI) broth in sterile universal tubes. Overnight cultures were grown at 37°C in a shaking (200 rpm) incubator. After overnight growth, each culture was diluted to an optical density at 600 nm (OD₆₀₀) value of 0.5 using phosphate-buffered saline (PBS). To 1 mL of the diluted culture, 1 mL of PBS (controls) or PBS containing 46% (v/v) isopropanol was added, and samples were vortexed for 5 seconds, followed by a 5-minute incubation at room temperature. Immediately following the incubation, mixtures were vortexed for 5 seconds and 100 µL was transferred to an Eppendorf tube containing 900 µL 7.5% (v/v) Tween 80 in PBS. This represented a 10⁻¹ dilution. A 10-fold dilution series in PBS was continued until a 10⁻³ dilution was obtained. 50 µL of each dilution was spread in duplicate onto BHI agar plates. Plates were incubated overnight at 37°C, and colonies were counted. The numbers of colony-forming-units per millilitre (CFU/mL) in treated samples were calculated by multiplying the number of colonies counted by the dilution factor, followed by the division of the resulting number by the volume plated.

PBS dilution series and spot plating

In a variant of the assay, treated samples were serially diluted in PBS. Samples were serially diluted between 10- and 10000-fold in PBS and 5 µL of each dilution was spot-plated in duplicate onto BHI agar plates.

2.2.2 Growth curve-based alcohol killing assay

Single *E. faecium* colonies were used to inoculate 10 mL BHI broth in sterile universal tubes. Overnight cultures were grown at 37°C in a shaking (200 rpm) incubator. After overnight growth, each culture was diluted to an OD₆₀₀ value of 0.5 using PBS. 100 µL of the diluted culture was treated with 100 µL of PBS (controls) or PBS containing isopropanol (concentrations tested: 11.5, 23, 34.5 and 46%), mixed and incubated for 5 minutes. Immediately before sampling, each culture was again mixed and 10 µL transferred into 190 µL of BHI broth in a Costar 96-well plate. Samples were incubated at 37°C for 19 hours in a Tecan spark microplate plate reader, and OD₆₀₀ measurements for each well were recorded every 15 minutes.

Growth parameters calculations

The obtained OD₆₀₀ measurements were plotted against incubation time to obtain growth curves. Since the 23% (v/v) isopropanol concentration consistently delayed cell growth in all strains it was used to calculate the percentage cell survival, following the isopropanol treatment. The growth curve plots were used to calculate the growth rate. Within each OD₆₀₀ axis the exponential phase was selected, and the scale converted into logarithmic (**Figure 2.2**).

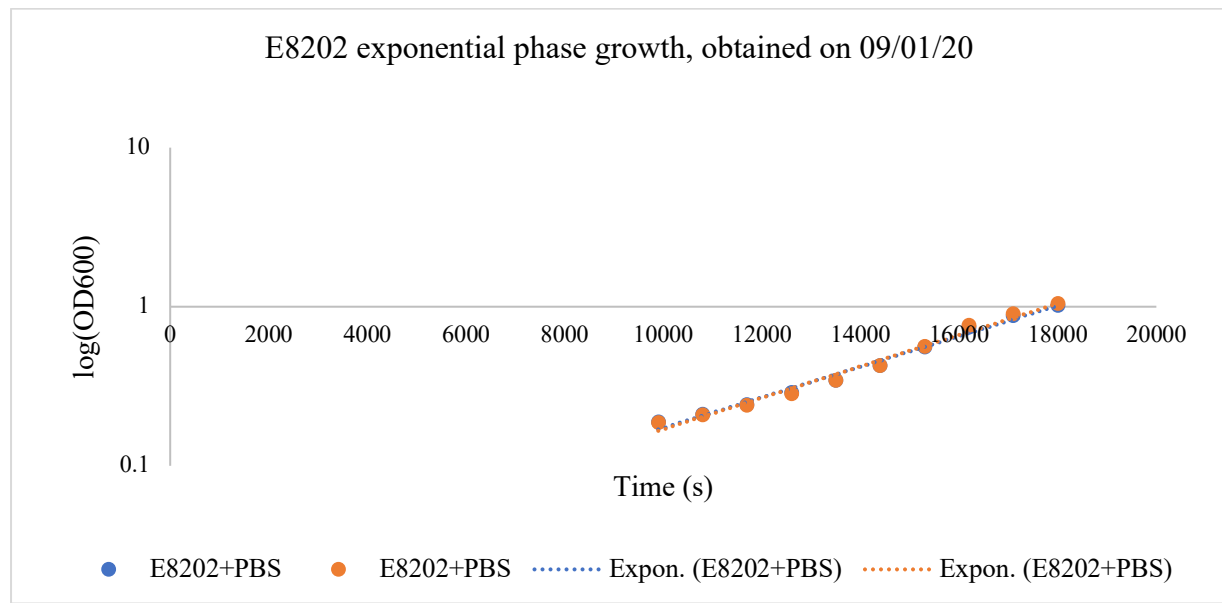


Figure 2.2: Example exponential phase selection, obtained for strain E8202.

The OD₆₀₀ and time (T) values corresponding to the beginning and end of the exponential phase in the PBS controls were used to calculate the growth rate (μ) for each of the analyzed replicates (**Equation 2.1**). This was followed by the calculation of the doubling time (t_d) (**Equation 2.2**)

Equation 2.1:

$$\mu = \ln \left(\frac{OD1}{OD2} \right) / (T2 - T1)$$

Equation 2.2:

$$t_d = \frac{\ln(2)}{\mu}$$

The time necessary for the 23% (v/v) isopropanol treated samples to reach an OD₆₀₀ density of 0.5, was subtracted from the time necessary for the PBS treated controls to achieve the OD₆₀₀ density of 0.5; in order to obtain the time difference (ΔT) between the two data points. ΔT was then divided by t_d to obtain x (**Equation 2.3**). Percentage survival in the samples treated with 23% (v/v) isopropanol was then calculated by multiplying 2 to the power of x.

Equation 2.3:

$$\frac{\Delta T}{t_d} = x$$

2.2.3 Rapid alcohol tolerance assay development

10 µL of SYBR Green I (Invitrogen) was mixed with 30 µL propidium iodide (20 mM, Sigma) into 1 mL of sterile dH₂O and vortexed thoroughly to prepare the 1:3 SYBRG:PI volume ratio solution. 30 µL of SYBR Green I (Invitrogen) was mixed with 10 µL propidium iodide (20 mM, Sigma) into 1 mL of sterile dH₂O and vortexed thoroughly to prepare the 3:1 SYBRG:PI volume ratio solution. Single *E. faecium* colonies were used to inoculate 10 mL BHI broth in sterile universal tubes. Overnight cultures were grown at 37°C in a shaking (200 rpm) incubator. 10 µL of the overnight cultures were used to inoculate 10 mL of BHI and incubated at 37°C until an OD₆₀₀ value of around 0.5 was achieved. 10 mL of each culture was centrifuged at 4000 rpm for 1 min, 9 mL of the BHI medium was removed and the pellet was resuspended in 9 mL PBS.

Method development

100 µL of the bacterial culture in PBS was treated with 100 µL of PBS (control) or PBS containing isopropanol (concentrations tested: 11.5, 23, 34.5, 46, 60 and 70%) in a Costar 96-well plate and mixed. The staining mixture (10 µL) was added to each well and mixed thoroughly. The plate was incubated at room temperature in the dark for 15 minutes. With excitation wavelength at 485 nm, the fluorescence intensities at 535 nm (green emission) and 635 nm (red emission) were measured for each well of the plate using a Tecan spark microplate plate reader.

Incorporation of a further dilution step

100 µL of the bacterial culture in PBS was treated with 100 µL of PBS or PBS containing isopropanol (concentrations tested: 34.5, 46, 60 and 70%) in a Costar 96-well plate. The samples were mixed, followed by a 5-minute incubation at room temperature. Immediately before sampling, each culture was again mixed and 10 µL transferred into 190 µL of PBS. The staining mixture (10 µL) was added to each well and mixed thoroughly. The plate was incubated at room temperature in the dark for 15 minutes. With excitation wavelength at 485 nm, the fluorescence intensities at 535 nm (green emission) and 635 nm (red emission) were measured for each well of the plate using a Tecan spark microplate plate reader.

2.3 Results

2.3.1 Alcohol killing assay incorporating the Miles and Misra method

The alcohol tolerance assessment conducted by Pidot *et al.* was based on the calculation of colony-forming units (CFU), following a 5-minute alcohol incubation and serial dilutions [54]. We have incorporated this method within our study and selected strains E8202 and E745 to be evaluated. Both strains are clinical isolates, classified as clade A1, indicating their high environmental adaptability and potentially high alcohol tolerance [55], [56].

Table 2.1: CFU/mL results obtained from the 5-minute treatment of *E. faecium* strains E745 and E8202 with 23% (v/v) isopropanol in PBS and the following serial dilutions in 7.5% Tween 80 in PBS.

n=average of two replicates per data point.

	E745 CFU/mL	E8202 CFU/mL	PBS treated control CFU/mL
Experimental replicate 1	2200	1800	All lawn
Experimental replicate 2	3600	15400	All lawn
Experimental replicate 3	All lawn	4800	All lawn
Experimental replicate 4	29000	4900	All lawn
Experimental replicate 5	All lawn	All lawn	All lawn
Experimental replicate 6	All lawn	21000	All lawn

The results obtained were inconclusive (**Table 2.1**). While in some replicates the cell count was visibly reduced by the addition of 23% (v/v) isopropanol, both strains occasionally exhibited seemingly full tolerance, showing a cell count comparable to the PBS treated controls. This was seen for 50% of E745 experiments (replicates 3, 5 and 6) and for replicate 5 of E8202. These results could be caused by a higher alcohol tolerance being present within E745, however the replicates yielding countable colonies provided similar values for both the strains, negating that theory.

Tween 80 has been reported to neutralise the disinfecting power of alcohol [57]. It was stipulated to have caused the high survival observed in some of the replicates in **Table 2.1**. The method was modified by replacing the 7.5% Tween 80 in PBS solution, used for serial

dilutions, with pure PBS (**Table 2.2**). Initially this modification yielded promising results in replicates 1-3, of both strains, which all showed comparable values. Interestingly the CFU/mL results for replicates 1-3 were higher than observed in the samples diluted in 7.5% Tween80, negating the theory that it inhibited the bactericidal activity of the alcohol. Despite the promising data seen in replicates 1-3, replicates 4-5 showed no cell survival.

Table 2.2: CFU/mL results obtained from the 5-minute treatment of *E. faecium* strains E745 and E8202 with 23% (v/v) isopropanol in PBS and the following serial dilutions in PBS.

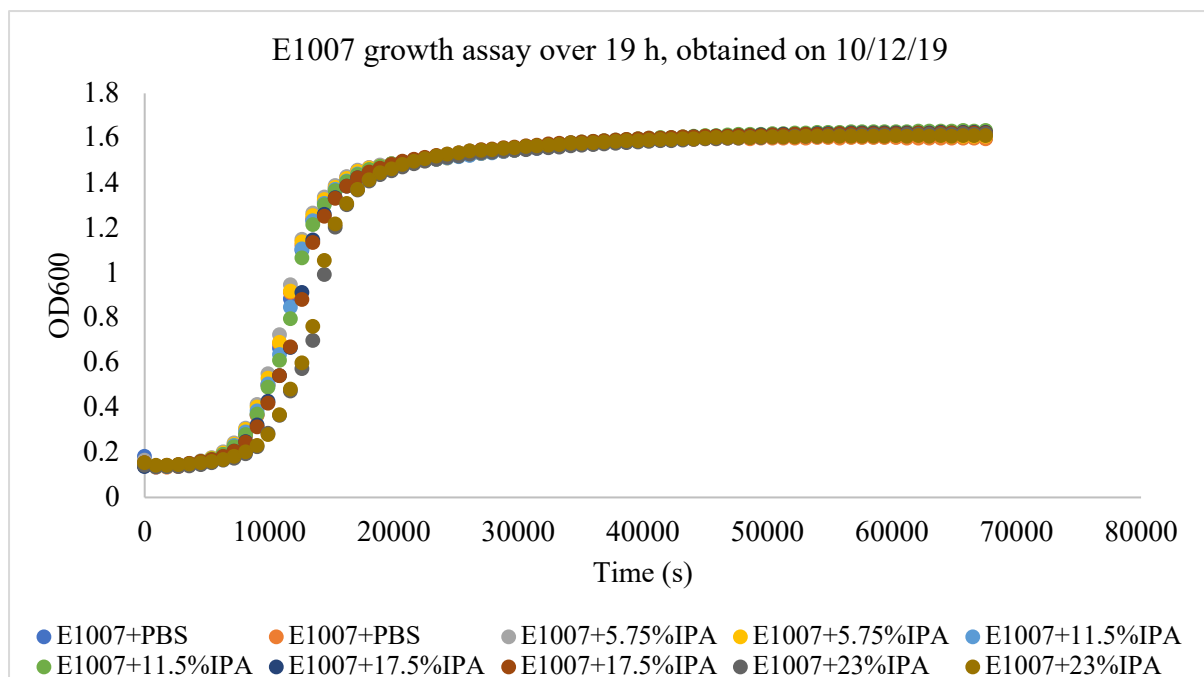
n=average of two replicates per data point

	E745 CFU/mL	E8202 CFU/mL	PBS treated control CFU/mL
Experimental replicate 1	54 x 10 ⁶	34 x 10 ⁶	All lawn
Experimental replicate 2	52 x 10 ⁶	18 x 10 ⁶	All lawn
Experimental replicate 3	42 x 10 ⁶	30 x 10 ⁶	All lawn
Experimental replicate 4	0	0	All lawn
Experimental replicate 5	0	0	All lawn

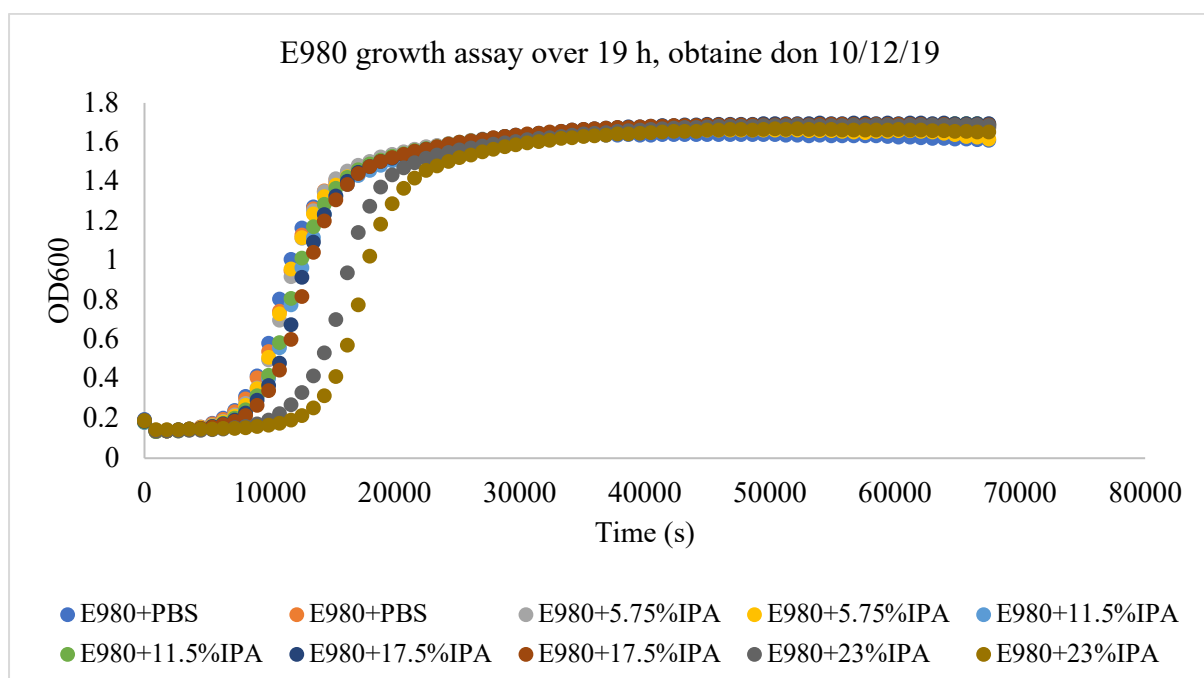
The reoccurring irreproducibility we had encountered using the Miles and Misra method exhibited a need for a new, more reliable assay for the testing of alcohol tolerance in *E. faecium*.

2.3.2 Growth curve-based alcohol killing assay

A limitation of the previously applied Miles and Misra method was the lack of optical density (OD₆₀₀) measurements, following the alcohol treatment and serial dilutions. Considering the repeatable inconsistencies, seen in the Miles and Misra assay results, an understanding of the changes in the cultures optical density, in the hours following the alcohol treatment, could prove highly useful. The application of a growth-curve based approach measuring the turbidity of cultures recovering from alcohol treatment, over the span of 19 hours, solved this issue [58]. OD₆₀₀ measurements were taken in 15-minute intervals and plotted against time to obtain growth curves for each strain (**Figure 2.3**). The exponential phase growth was then used to calculate the percentage of survival for each of the analysed strains.



a)



b)

Figure 2.3: Example growth curves, obtained for *E. faecium* strains E1007 (a) and E980 (b).

Previously analysed E8202 and E745 were included in the study, with an additional A1 clinical strain - E7345 being incorporated [59]. Furthermore, E1007 and E980 - strains of human commensal origin, belonging to clade B and exhibiting low levels of antibiotic resistance were chosen [60], [47]. Additionally, recently isolated clinical strains LVRE01 and LVRE03 were included, to evaluate the potential increase of alcohol tolerance in newly obtained clinical

strains. Lastly, E688 a strain of animal origin and low antibiotic resistance was chosen [47]. This diverse set of strains of different origin, mutation rate capabilities and antibiotic resistance recorded, was selected to obtain a detailed understanding of alcohol tolerance currently present within the environment.

The selected strains were incubated with a range of alcohol concentrations (0, 5.75, 11.5, 17.25 and 23%), over a period of 5-minutes, following which they were diluted in BHI, and their optical density measured for 19 h. The majority of isolates, excluding strain E7345, showed tolerance to the lower and medium alcohol concentrations (0%, 5.75%, 11.5%, 17.25%), with only the 23% (v/v) isopropanol treatments visibly delaying culture growth. Since the 23% (v/v) isopropanol concentration consistently delayed cell growth in all strains, its exponential phase data was used to calculate the percentage cell survival (**Figure 2.4**).

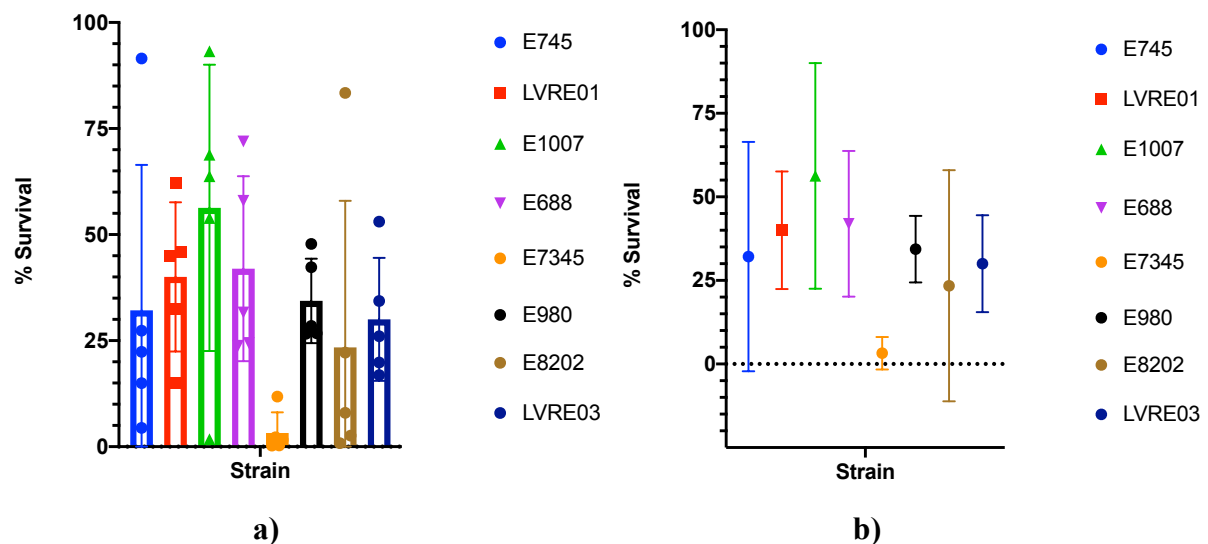


Figure 2.4: *E. faecium* percentage cell survival after a 5-minute treatment with 23% (v/v) isopropanol and a following 1:19 dilution in BHI **a)** individual replicate data and average **b)** mean value and error.

n= average of two replicates per data point

E745 and E8202 showed the highest variability out of the eight strains analysed, which could explain the inconsistent results obtained using the Miles and Misra method. Strains showing higher reproducibility (E980, LVRE03 and E7345) were selected to validate that hypothesis (**Table 2.3**).

Table 2.3: CFU/mL results obtained from a Miles and Misra assay of *E. faecium* strains E980, E7345 and LVRE03 with 23% (v/v) isopropanol in PBS for 5 minutes and the following serial dilutions in PBS. The PBS treated control for each experiment showed full survival (all lawn).

n= average of two replicates per data point

	E7345 CFU/mL	E980 CFU/mL	LVRE03 CFU/mL	LVRE03 CFU/mL
Experimental replicate 1	22 x 10 ⁶	10 x 10 ⁷	42 x 10 ⁶	14 x 10 ⁶
Experimental replicate 2	12 x 10 ⁶	66 x 10 ⁶	6 x 10 ⁶	8 x 10 ⁶
Experimental replicate 3	24 x 10 ⁶	48 x 10 ⁶	4 x 10 ⁶	0
Experimental replicate 4	20 x 10 ⁶	14 x 10 ⁶	0	0

The CFU/mL data obtained for E980 and E7345 correlates well with the results obtained in the growth curve-based assay, showing a higher alcohol tolerance for E980 in the majority of replicates. LVRE03 showed anomalous low survival for one of its replicates, as seen in the third column of **Table 2.3**. For that reason, the assay of LVRE03 was repeated, this time yielding two anomalous results of no survival. While the Miles and Misra assay results for E7345 and E980 were consistent, the variability seen for LVRE03 raised similar irreproducibility issues as seen previously for E745 and E8202.

2.3.3 Rapid alcohol tolerance assay development

While the growth curve-based assay provided reproducible results, a rapid assay for determining alcohol tolerance is highly coveted and was further investigated. We have applied a combination of fluorescent dyes: SYBR green (SYBRG) and propidium iodide (PI) to assess the number of permeable/damaged cells produced by an isopropanol treatment of strain E980 [61],[62]. E980 was selected based on its consistent results of moderate alcohol tolerance in the growth-curve based assay.

PI can only cross compromised bacterial membranes and stains DNA and RNA inside of dead or damaged cells resulting in the emission of a red fluorescence signal [63]. In the following assay we coupled PI with SYBRG - a universal stain that crosses intact membranes and stains nucleic acids in all cells. PI has been reported to possess a higher affinity for DNA, than

SYBRG, when both stains are exposed to the same nucleic acid, resulting in a red fluorescent signal and enabling the assessment of alcohol damage within the bacterial population [64].

Since no reports were found regarding the optimal staining protocol for enterococci, we have adapted a method reported to work on *Borrelia burgdorferi* [61]. Taking into account different dye ratios reported to work on different species we evaluated two volume combinations: 1:3 PI:SYBRG and 3:1 PI:SYBRG [62]. Overnight cultures (OD₆₀₀ around 4) were assessed, together with cultures of OD₆₀₀ values around 0.5. Preliminary tests of this assay indicated a high detection limit may be necessary for the fluorescence measurements. For that reason, to ensure a sufficiently high cell count was present in the early stages of method development, a dilution step was omitted following the alcohol exposure. This resulted in an excess 15-minute alcohol incubation time (seen in **Figures 2.4** and **2.5**), compared to the 5 minutes applied in the previous assay methods. The 15-minute alcohol incubation resulted from a necessary fluorescent dye incubation time of 15 minutes, which had to be incorporated within the method.

A preliminary study showed the BHI broth, initially used to suspend cells, produced a strong background intensity. The method was then modified by replacing the BHI medium with PBS, proven to produce little background noise (**Table 2.4**).

Table 2.4: Fluorescence intensity for blank BHI and PBS samples, tested with the 3:1 PI:SYBRG volume ratio.

	SYBRG fluorescence intensity (RFU)	PI fluorescence intensity (RFU)
BHI	27616/27724	43954/43711
BHI	27765/28536	41875/43915
PBS	90/89	6296/6068
PBS	94/104	6500/6383

The initial method development assays were conducted using 5.75, 11.5, 17.25 and 23% (v/v) isopropanol concentrations (**Figure 2.4**). The PI intensity gave comparable results for all the treatment conditions, including the PBS treated controls. A slight increase in the PI intensity in cultures with OD₆₀₀ values of around 4, treated with 23% (v/v) isopropanol was observed, however it was not as high as expected. This lack of change between treatment conditions, seen

for the PI data, does not fit well within literature predictions, which indicated a gradual increase of PI intensity would take place as the isopropanol concentration was enhanced [61]. SYBRG data showed a significant intensity decrease, as the isopropanol percentage increased to 23% (v/v) for both the OD₆₀₀ values and dye combinations analysed (**Figure 2.5**). The higher OD₆₀₀ value resulted in a higher fluorescent intensity and was more consistent.

Out of the two dye combinations tested, the 1:3 PI:SYBRG produced more coherent results between the data points of both the OD₆₀₀ cultures. This was in particular visible for the cultures with a lower optical density of around 0.5. The 1:3 PI:SYBRG combination was selected for future assays.

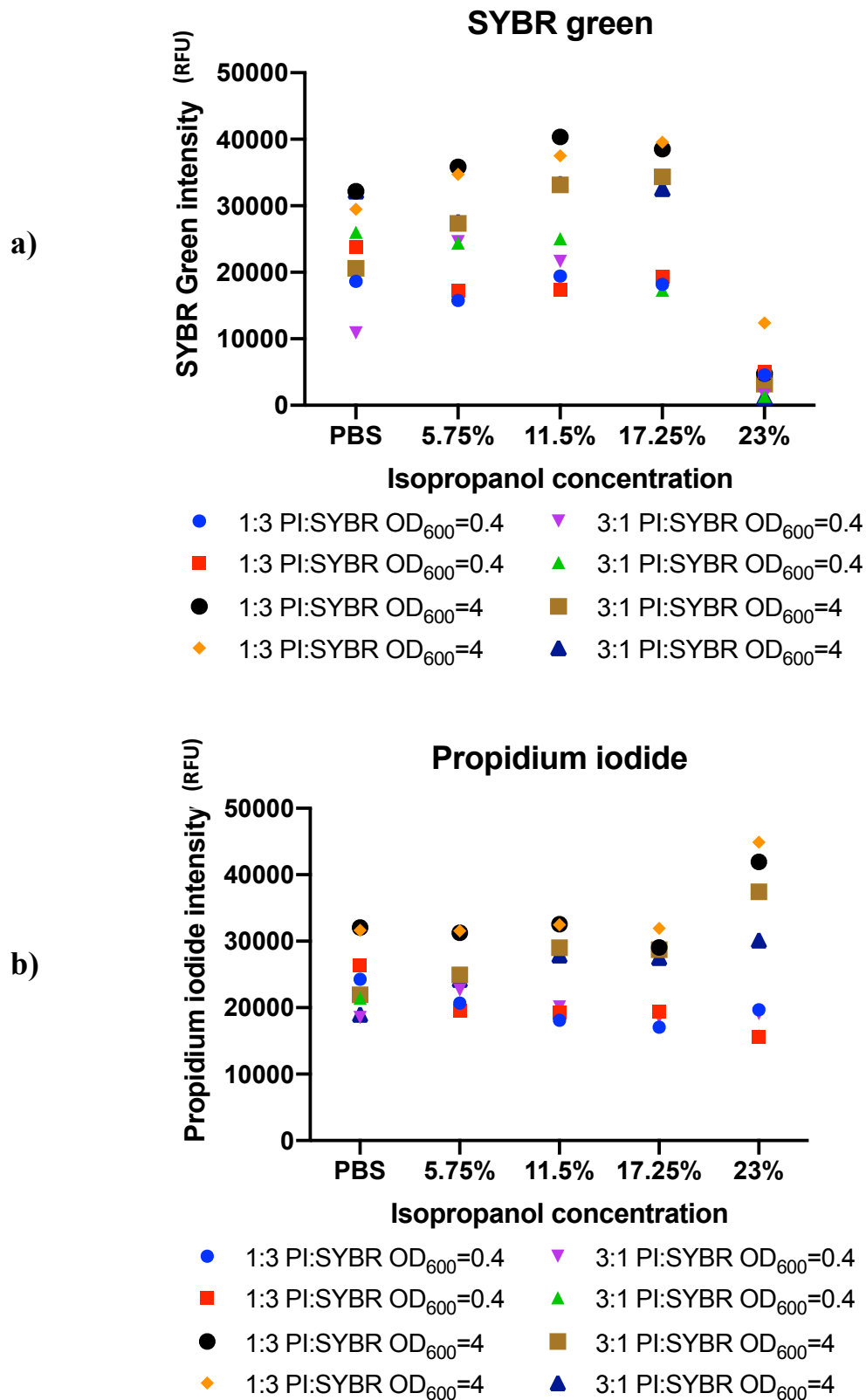


Figure 2.5: SYBRG (a) and PI (b) fluorescent intensity for *E. faecium* E980 cultures treated 50:50 with isopropanol in PBS or PBS for 15 minutes.

n= average of two replicates per data point

The substantial intensity change, observed for the SYBRG data at the 23% (v/v) isopropanol concentration was deemed promising, despite the inconclusive results seen for PI. It was stipulated the comparable results seen for the lower alcohol concentrations (5.75, 11.5 and 17.25%) could result from an insufficiently harsh alcohol treatment. The isopropanol concentrations tested further were increased to 17.25%, 23%, 30% and 35% (v/v) (**Figure 2.6**).

Following the heightening of the alcohol concentrations tested, the PI data exhibited a decline in intensity, as the isopropanol concentration increased, contrasting with previous results seen in **Figure 2.5**. SYBRG showed results similar to those seen in **Figure 2.5**, with its fluorescent intensity decreasing, as the isopropanol concentration increased. A SYBRG intensity spike was observed for the 17.25% (v/v) treatment condition, for cultures with OD₆₀₀ values of 0.5, while the cultures with OD₆₀₀ values of 4 showed an intensity decrease. The remaining treatment conditions showed comparable SYBRG data for both the OD₆₀₀ values analysed.

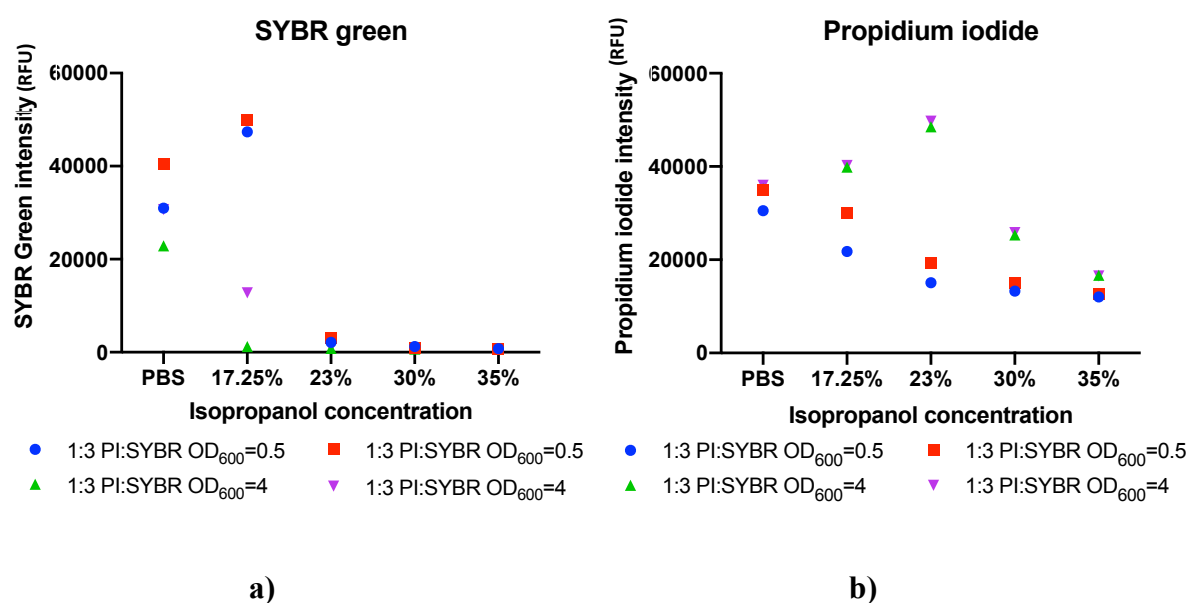


Figure 2.6: SYBRG (a) and PI (b) fluorescent intensity for E980 cultures treated 50:50 with isopropanol in PBS or PBS for 15 minutes.
n= average of two replicates per data point

As mentioned previously, with the objective of maintaining a high cell count throughout the early stages of method development an excess alcohol incubation time of 15 minutes was so far applied. This was caused by an elimination of a dilution step, following the alcohol treatment and previous to sample incubation with the fluorescent dye. As the SYBRG data obtained so far showed reproducible results, a further dilution step was incorporated, after a

5-minute alcohol treatment (**Figure 2.7**). The dilution step was followed by a 15-minute incubation with fluorescent dyes.

Again, the SYBRG data showed a gradual decrease in intensity recorded, as the isopropanol concentration increased (**Figure 2.7**). This was observed for both the OD₆₀₀ values analysed, with a stronger gradient being observed in the higher optical density culture. The more reproducible results, seen throughout this method development for the higher OD₆₀₀ cultures, indicate an optical density value of around 0.5 may be too dilute to accurately assess the alcohol tolerance present. Nonetheless further research is required to fully evaluate this hypothesis. The PI results showed an intensity increase as the isopropanol concentration increased, as previously observed in **Figure 2.5**. While the PI data so far is insufficient to determine its effectiveness, the SYBRG results show the potential of this rapid staining assay, when assessing the alcohol tolerance in *E. faecium*. However, further method development is required to precisely define the OD₆₀₀ detection limit and to explain the fluctuation observed for the PI data.

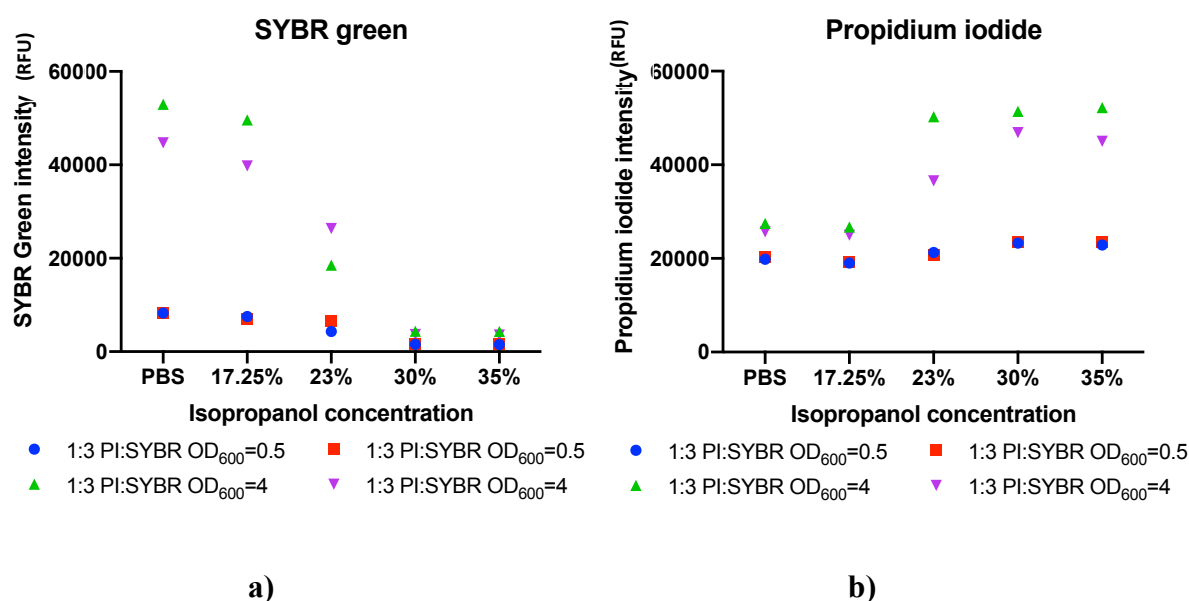


Figure 2.7: SYBRG (a) and PI (b) fluorescent activity for E980 cultures treated 50:50 with isopropanol in PBS or PBS for 5 minutes, followed by a 1:19 dilution in PBS.

n= average of two replicates per data point

2.4 Discussion

Out of the eight strains analysed, E8202 and E745 showed the highest survival variability, exhibiting both high resistance and high susceptibility. The lowest tolerance to the 23% (v/v) isopropanol concentration was observed for E7345, while E1007 showed the highest resistance, with one anomalous result of low survival being observed. Surprisingly, the recently isolated clinical strains LVRE01 and LVRE03 showed only moderate tolerance to 23% (v/v) isopropanol, indicating alcohol tolerance has not progressed further in the clinical environment. Strains of commensal and animal origin (E688, E980 and E1007) displayed high isopropanol resistance indicating alcohol tolerance is not correlated with clinical origin. All the analysed strains, excluding the highly susceptible E7345, exhibited a survival rate higher than 20%, when treated with 23% (v/v) isopropanol, showing moderate alcohol tolerance is a commonly occurring phenomenon. While the 23% (v/v) concentration is significantly lower than the recommended isopropanol concentrations for clinical use (70-90%), Pidot *et al.* have demonstrated tolerance to lower alcohol concentrations enhances the likelihood of transmission, when subjected to a full 70% (v/v) alcohol solution [⁵⁴].

Interestingly, the results obtained show alcohol tolerance is not directly correlated with clade classification. Clade A1, which is predominantly responsible for the high antibiotic resistance levels seen in *E. faecium* populations, showed either high or medium susceptibility to the 23% (v/v) isopropanol treatments. Moreover, strain E7345, classified as clade A1, exhibited the highest susceptibility of all the strains analysed. Other A1 strains: E745 and E8202 showed variable results and did not display higher tolerance than seen for isolates belonging to clades A2 and B.

Throughout the study significant issues arose from the incorporation of the Miles and Misra method. The high irreproducibility seen for this method could be caused by human error, however the assay had been attempted by other group members once again yielding inconsistent results. Furthermore, the small number of replicates applied within this study could be an additional cause of the high variability observed. While Pidot *et al.* have reported it as an effective method for measuring alcohol tolerance, their method was more automated, reducing the risk of variation caused by human error and environmental factors.

The rapid assay incorporating SYBRG and PI shows potential, in particular for the analysis higher optical density cultures, through the evaluation of SYBRG intensity changes. The anomalous results seen for PI could result from the gram-positive cell wall structure of *E. faecium*, as the method applied was mostly tested on gram-negative bacteria [61],[62]. Intact cells of *Bacillus clausii* reportedly had the same red fluorescence intensity, as that of permeabilized cells and differed only in green fluorescence intensity [65]. This contrasts with the data seen for *Lactococcus lactis* where equal green fluorescent intensity but differences in the red fluorescence were detected [66]. The above examples indicate gram-positive bacteria behave unpredictably when treated with fluorescent dyes. Further research is required to evaluate the inconsistencies seen for the PI data. Furthermore, a precise detection limit must be established, and the methodology tested on *E. faecium* strains other than E980.

2.5 Conclusions

Increasing alcohol tolerance observed within *E. faecium* in recent years poses a substantial threat to the current disinfection strategies. We have evaluated the alcohol tolerance present in strains of clinical, animal and human commensal origin, using three different assay methods. The data obtained shows alcohol tolerance has not progressed further within the clinical setting with the highest tolerance to 23% (v/v) isopropanol being observed in human commensal strains. While our study does not show the progression of isopropanol tolerance in clinical strains the number of isolates analysed was small and is not fully representative of alcohol tolerance present in the clinic. Seven out of the eight strains analysed exhibited moderate tolerance to the 23% (v/v) isopropanol treatment, showing that while alcohol tolerance has not progressed further within the clinic, it is a common issue that is likely to grow in importance. No correlation between the MDR, clinical clade A1 and alcohol tolerance present was observed. Alcohol tolerance assays based on the creation of a growth curve using OD₆₀₀ measurements, taken over a time span of 19h, have been validated as an effective strategy to precisely evaluate the tolerance present. Furthermore, we have shown the high potential of a rapid assay, based on the use of fluorescent dyes, however further method development is required establish an accurate detection limit and understand the variation observer for the PI results.

3. Genomic analysis of resistance genes present in 63 *E. coli* isolates from community hospitals in North Carolina.

Abstract

Extended spectrum β -lactamases are enzymes produced primarily by the Enterobacteriaceae, in particular *Klebsiella pneumoniae* and *Escherichia coli*. The antimicrobial resistance correlated with these enzymes, poses an increasing threat to public safety. This is particularly the case for *E. coli* clone ST131, shown to rapidly spread in community and healthcare settings since 2000. To understand and control the dissemination of MDR clones, continuous surveillance of ESBL-producing Enterobacteriaceae is necessary. Whole genome sequencing enables the investigation of bacterial gene transmission among host populations and allows a comprehensive analysis of AMR present in the community and clinic. We have performed a comparative analysis of 63 *E. coli* genomes, obtained from community hospitals in North Carolina, by Kanamori *et al.* [67]. The results show a dominance of clone ST131 within the analysed population (62%). Within ST131, the multidrug-resistant subclone H30-Rx accounted for the majority of isolates (69%). ESBL type CTX-M-15, commonly associated with H30-Rx, was most common within the analysed isolates. Resistance to current antimicrobial therapies was observed throughout all the isolates, with the highest resistance percentages being observed within H30-Rx, showing the wide-spread propagation of MDR *E. coli* subclones in North Carolina.

3.1 Introduction

Enterobacteriaceae are common causes of community and hospital-acquired infections. The high prevalence of these organisms, combined with their excellent genetic plasticity, poses an growing threat to public health [68]. Of particular concern is the increasing frequency of ESBL-producing Enterobacteriaceae [69]. ESBL production, enables the deactivation of β -lactam antibiotics and is frequently combined with co-resistance towards aminoglycosides and FQs, resulting in the elimination the key classes of antibiotics used on gram-negative species. Carbapenems, typically reserved for treating highly resistant infections, must now be employed to treat ESBL-producing Enterobacteriaceae infections, however the increasing rise in carbapenem resistance poses a threat to this last-resort treatment strategy [68].

3.1.1 CTX-M type ESBLs

ESBLs were first described in 1983. The activity observed within the first reported isolates, resulted from the production of a variant of the SHV-1 enzyme, found in *Klebsiella pneumoniae*. This was followed by the reporting of variants of TEM-1 and TEM-2 enzymes, showing similar substrate and inhibition profiles to those of SHV-1 derivatives [70]. These ESBL types conferred resistance to broad spectrum penicillins, but could not inhibit the action of oxyimino cephalosporins [71]. TEM-1, TEM-2 and SHV-1 have accounted for the majority of clinical cases identified during the 1980s and 1990s [72], however the dominant type of ESBL expressed by the Enterobacteriaceae has changed in recent years [73]. Since 2000 a dramatic increase in the numbers of CTX-Ms enzymes and organisms producing them has been reported, making them the most prevalent ESBLs worldwide [69], [70]. This is particularly the case among *E. coli*, which has replaced *Klebsiella* spp. as the principal ESBL-producing member of the Enterobacteriaceae [67] (**Figure 3.1**). Compared to other ESBL types CTX-Ms show extended-spectrum activity against broad spectrum β -lactams, linked to the presence of Serine-237 within their structure [74].

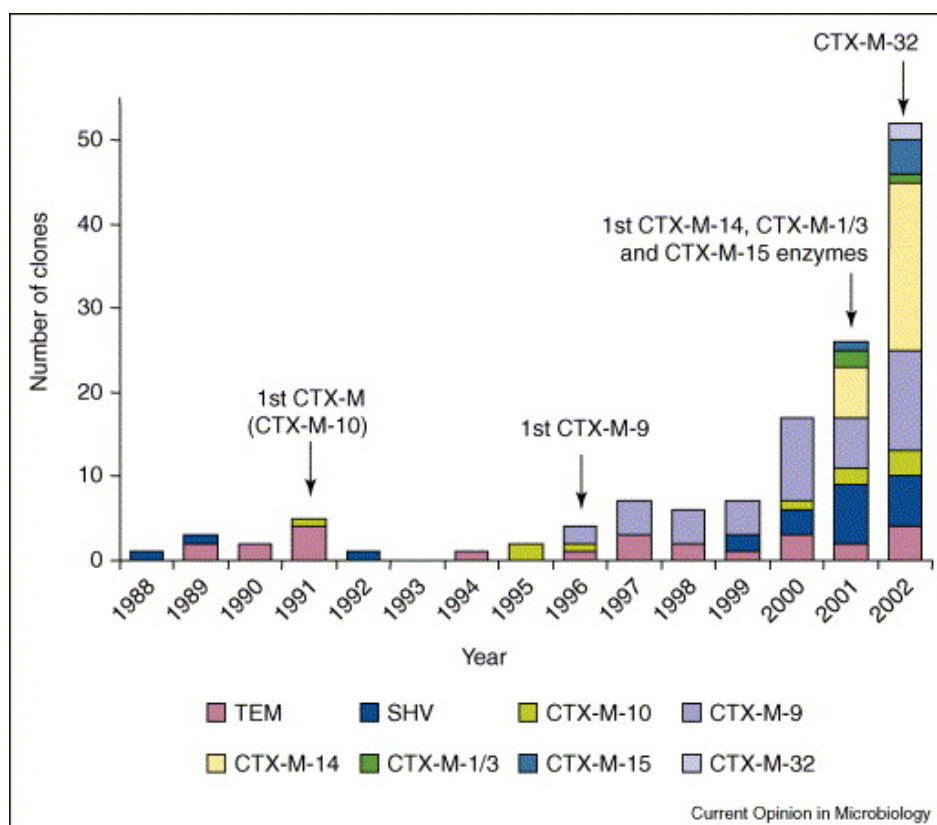


Figure 3.1: Shifting epidemiology of different ESBL-producing *E. coli* isolates from 1988 to 2002 at the Ramón y Cajal University Hospital in Madrid. Image taken from [70].

CTX-M genes originate from the genus *Kluyvera*, comprising of a number of environmental species, with little or no pathogenic activity against humans [75]. The spread of the CTX-M-type enzymes, among the rest of the Enterobacteriaceae, is stipulated to originate from the capture of *bla*_{CTX-M} genes, from varying sources within the *Kluyvera* genus. The genes were then spread horizontally through self-conjugative plasmids, often carrying additional resistance determinants [75]. The rapid globalization of CTX-M could have been originated from a range of factors such as; an increase in international travel, presence of CTX-M in uncooked food products transported from one country to another and CTX-M dissemination within companion and wild animals [76]. CTX-M enzyme types vary based on the geographic area, as seen for CTX-M-2, until recently largely predominant in South America. Nevertheless, since 2009 an increasing dominance of the MDR CTX-M-15 type can be observed world-wide (**Figure 3.2**).

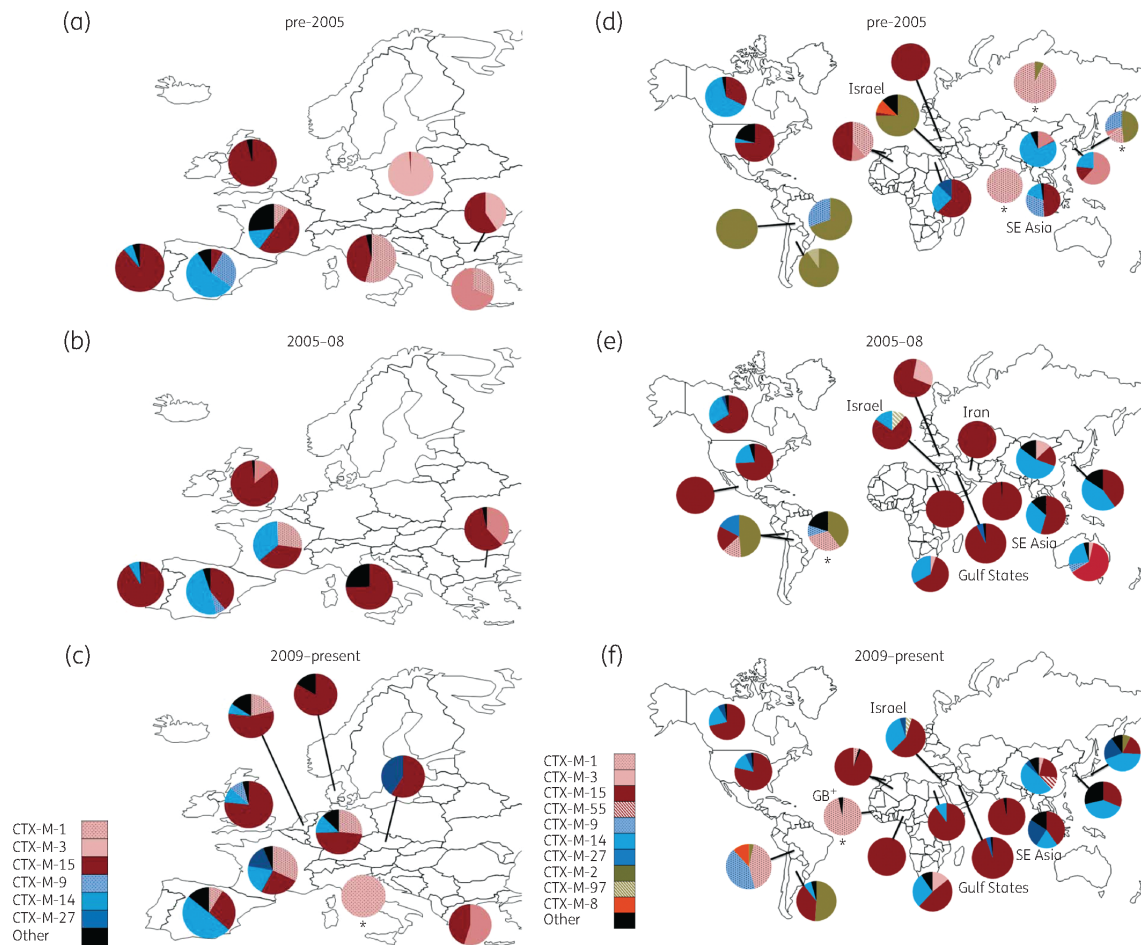


Figure 3.2: (a), (b), (c) CTX-M trends over three time periods for Europe (data include both hospital and community isolates) (d), (e), (f) CTX-M trends over three time periods for the rest of the world (data include both hospital and community isolates). South-East (SE) Asia includes Cambodia, Indonesia, Malaysia, The Philippines, Taiwan and Vietnam. Gulf States include Kuwait, Saudi Arabia and United Arab Emirates. Data for Australia and New Zealand are combined. GB⁺, Guinea-Bissau.

Note: many countries have no published data for certain time periods.

Image taken from [77].

The rapid dissemination of CTX-M is especially visible in the United States, where until 2007 TEM- and SHV-type ESBLs accounted of the majority of cases. Recent studies have however shown CTX-M enzymes have now become predominant, mostly through the emergence of CTX-M-15 [78]. The number of ESBL-producing Enterobacteriaceae cases in the US has been shown to consistently increase since 2012, with almost half the infections being community-associated [21]. According to the CDC, ESBL-producing Enterobacteriaceae accounted for

nearly 200 thousand infections in 2017, resulting in an estimated 1.2 billion dollars of healthcare costs [21].

3.1.2 *E. coli* clone ST131

The sudden worldwide increase of CTX-M producing *E. coli* has been closely associated with a single clone represented as sequence type ST131, identified using multilocus sequencing typing (MLST) [79]. ST131 is now the cause of millions of AMR infections annually and is predominantly associated with the spread of FQ and cephalosporin resistance [80]. In addition to their high resistance profiles, ST131 isolates are equipped with a rich library of virulence-associated genes, making them highly pathogenic and a common cause of community- and hospital-acquired infections [81].

Similar, to other *E. coli* clones ST131 harbours the *fimH* gene (encoding type 1 fimbrial adhesin) [81]. Through the analysis of 352 subclonal structures of ST131, Johnson *et al.* observed seven *fimH*-based clonal lineages within the ST131 clonal population, with *fimH30* being most prevalent. The H30 lineage originating from the *fimH30* allele has been considerably associated with FQ resistance, through its subclone H30-R [82]. Banerjee *et al.* reported H30 strains exhibit a close genetic similarity, suggesting they all arose from a single *fimH30*-carrying ancestor. These findings indicate that the rapid emergence of FQ resistant ST131 strains had been driven by clonal expansion and dissemination rather than by independent acquisition of FQ resistance genes in heterogeneous strains [83]. Recently, a new H30-R sublineage, named H30-Rx, has been observed by Price *et al.* Strains within this sublineage carry CTX-M-15 genes, which combined with FQ resistance inherent for all H30-R clones, results in their dramatic resistance capabilities and rapid spread [83],[84] (**Figure 3.3**).

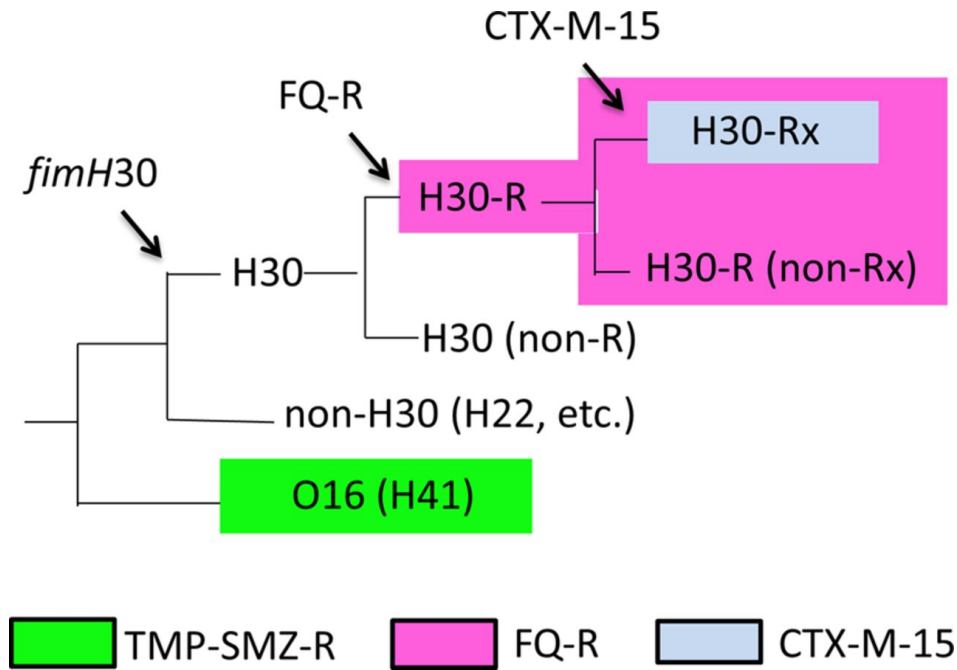


Figure 3.3: Schematic dendrogram of ST131 phylogeny. Arrows indicate emergence of *fimH30* allele, FQ resistance, and CTX-M-15 ESBL. Colours indicate resistance traits significantly associated with (although not confined to) specific lineages. FQ, fluoroquinolone; TMP-SMZ, trimethoprim-sulfamethoxazole; R, resistance. Image taken from [83].

The prevalence of clone ST131 has been shown to rapidly increase since 2000 [79]. Global surveillance of *E. coli* community and hospital populations is required to control the dissemination of this dangerous lineage. This is especially the case for FQ resistant H30-R and multidrug-resistant H30-Rx subclones, both posing a significant danger to patients. The genomic sequences of bacteria are an excellent source from which AMR can be accurately predicted [85]. Furthermore, whole-genome sequencing (WGS) and comparison enables the investigation of bacterial gene transmission among host populations, making it highly useful in clinical settings [86]. An interesting recent example of applying WGS in the tracking of ST131 has been reported by Kanamori *et al.* The group applied WGS to investigate 63 MDR *E. coli* isolates collected over the span of 6 years from 7 North Carolina community hospitals. Within the analysed isolates a high prevalence of ST131 was reported (62%) [67]. These observations provide an insight into MDR *E. coli* developments in North Carolina and exhibit the significant increase in ST131 populations in recent years. We have taken the 64 *E. coli* genomes provided by Kanamori *et al.* and used them to perform our own bioinformatic analysis, in order to verify their accuracy and assess the resistance present.

3.2 Materials and methods

64 genomes were obtained from NCBI using PRJDB4868 as the accession number. *De novo* genome assembly was conducted using SPAdes [104] and its quality verified using QUAST [105]. Contigs.fasta files were used to perform the bioinformatic examination. As no N's per 100 kpb were observed within the QUAST output the assemblies were used for further analysis. The genomes were annotated using Prokka [106], followed by the creation of a pangenome and core gene alignment using Roary [87]. The 63 *E. coli* isolates were mapped against a joined reference (DRR061493) using snippy [107], in order to obtain a high-resolution SNP-based tree. Phylogenetic trees were created using RAxML [88]. The phylogenetic trees were visualised using FigTree [108]. Multilocus sequence typing (MLST) was conducted in order to classify the isolates [109]. Resistance gene mapping was conducted using abricate [93]. Resistance and MLST data were visualised using Phandango [110].

3.3 Results

3.3.1 Phylogeny and pangenome analysis

To ensure all the isolates analysed belonged to *E. coli*, pan-genome analysis was applied, using Roary [87]. One isolate was deemed a different species (DRR061452), based on its dissimilar genetic profile (**Figure 3.4**). This isolate was then removed from further analysis reducing the total amount of isolates analysed to 63. As can be seen from **Figure 3.4**, the remaining isolates show close pangenome similarity. MLST analysis verified the remaining 63 isolates were indeed *E. coli* organisms, agreeing with the results of Kanamori *et al.* [67].

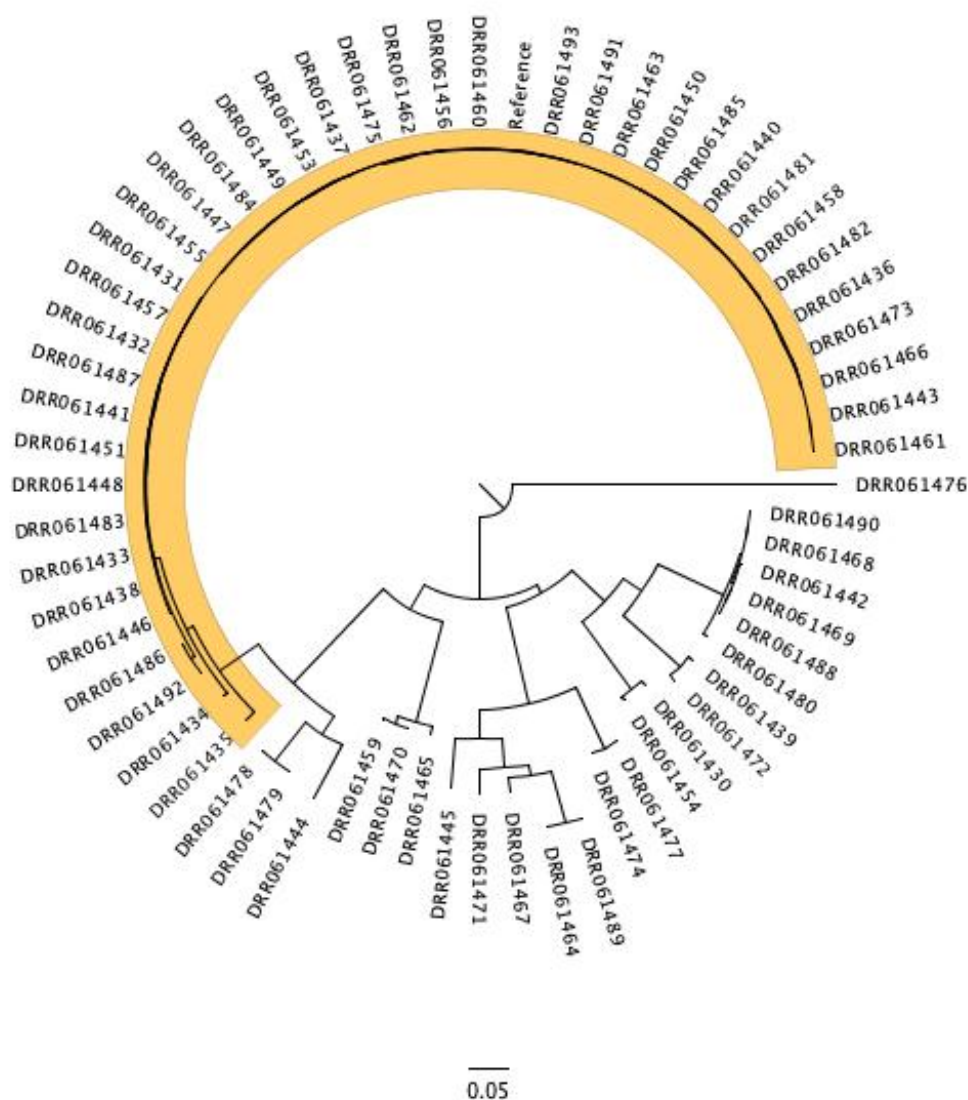


Figure 3.5: Phylogenetic tree of the analysed 63 *E. coli* isolates, obtained using RAxML, isolates highlighted in orange correspond to clone ST131.

Within the ST131 population the majority of isolates (95%) belonged to one, distinct subgroup (**Figure 3.6**, highlighted in green). This subpopulation was classified as H30-R, based on its FQ resistance reported by Kanamori *et al.* Further analysis showed the major group within this subpopulation is H30-Rx, accounting for 69% of the ST131 isolates, followed by 26% of ST131 isolates classified as non-H30-Rx. The two isolates not classified as H30-R (DRR061434, DRR061435) were reported to belong to subclones H30-S and H41 respectively, by Kanamori *et al.* [67]. H30-S is a FQ-susceptible H30 variant [84], while H41 is a commensal strain also showing FQ susceptibility [90], [91]. The results observed show H30-Rx has become predominant within *E. coli* populations in North Carolina.

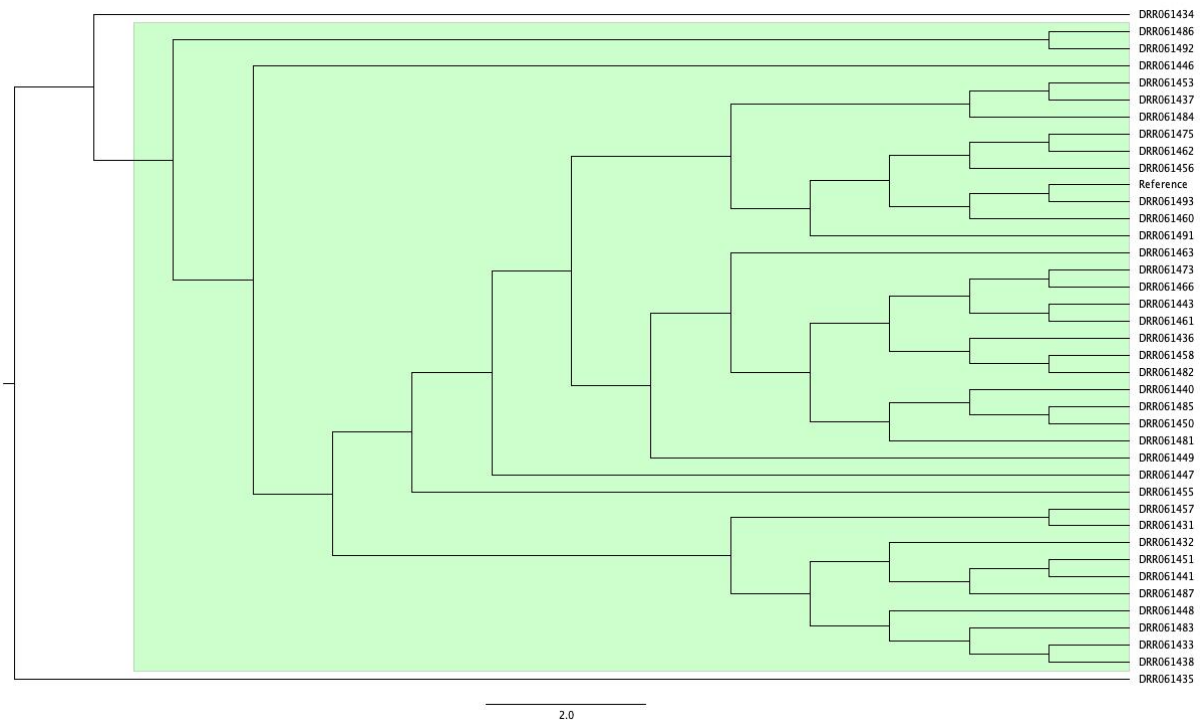


Figure 3.6: A cladogram of the 39 ST131 *E. coli* isolates, obtained using RAxML, mapped against isolate DRR061493 as a joint reference. Highlighted in green is the H30-R subclone group.

3.3.2 Presence of resistance genes

Antimicrobial susceptibility of *E. coli* isolates is an important clinical concern [92]. The presence of resistance genes in both ST131(**Figure 3.6**) and non-ST131 (**Figure 3.7**) isolates was assessed using abricate, enabling the mass screening of contigs for antimicrobial resistance or virulence genes [93]. On average ST131 isolates were shown to contain more antibiotic resistance genes than non-ST131 isolates. High resistance gene percentages were significantly associated with strain H30-Rx, supporting its association with high MDR. The results show the majority of ST131 isolates carry CTX-M-15, an ESBL currently most predominant world-wide alongside CTX-M-14. The emergence of these two ESBLs has been documented in the United States in recent years and is likely to progress further [94],[95],[96],[78]. CTX-M-15 isolates (26 detected) were significantly more common than CTX-M-14 (3 detected), showing CTX-M-15 and the H30-Rx the subclone associated with it, have become the dominant population in North Carolina (**Figure 3.8**).

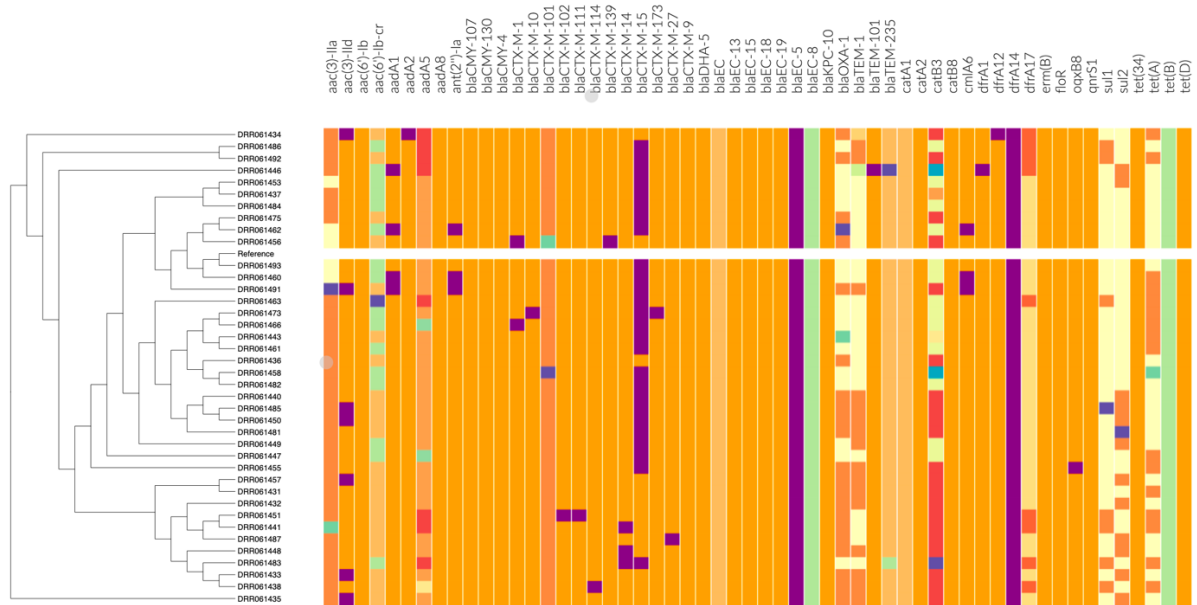


Figure 3.6: Resistance genes observed within the 39 ST131 isolates using abricate, where orange and red stand for gene absence and yellow, purple, blue, violet and green stand for either partial or full gene coverage; exceptions: for *aadA5* the peach orange stands for full coverage, for *blaTEM-1* and *sul2* orange means full coverage and yellow stands for no resistance gene detection, for *dfrA14* purple stands for no gene detection and for *tet(B)* and *blaEC-8* green stands for no gene detection.

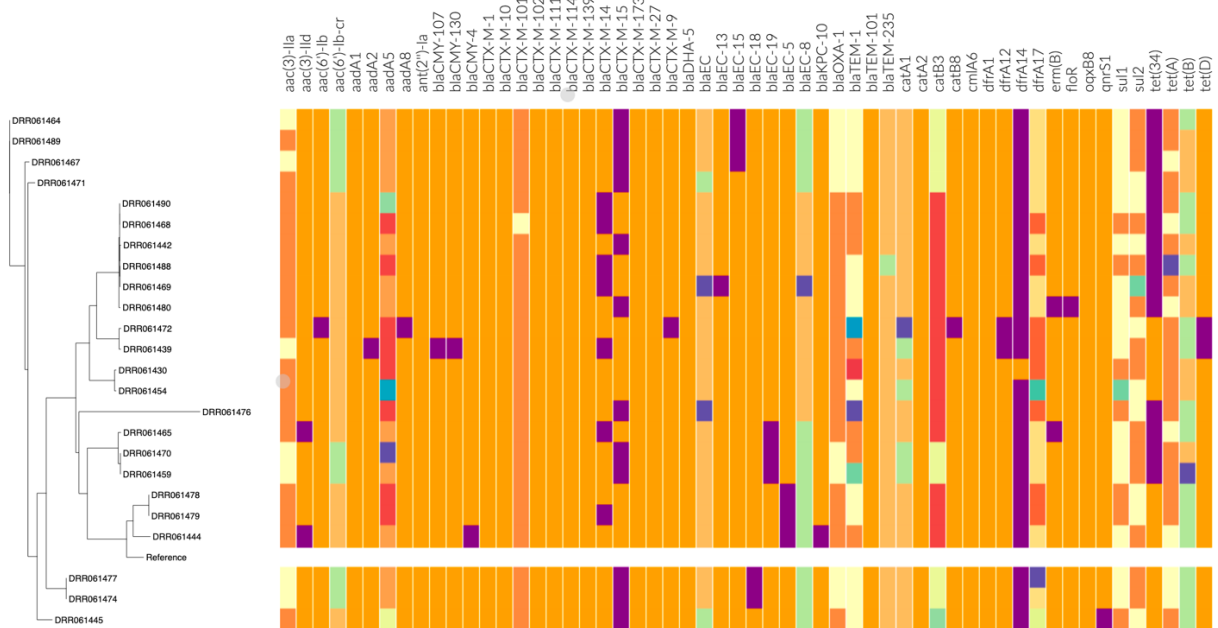


Figure 3.7: Resistance genes observed within the 24 non-ST131 isolates using abricate, where orange and red stand for gene absence and yellow, purple, blue, violet and green stand for either partial or full gene coverage; exceptions: for *aadA5* the peach orange stands for full coverage, for *blaTEM-1* and *sul2* orange means full coverage and yellow stands for no resistance gene detection, for *dfrA14* purple stands for no gene detection and for *tet(B)* and *blaEC-8* green stands for no gene detection and orange for full coverage.

3.3.2.1 CTX-M analysis

The presence of the CTX-M-15 gene was then determined in order to classify isolates as H30-Rx, within the ST131 clone (**Figure 3.8**). While the presence of CTX-M-15 is not a defining determinant when assigning isolates to H30-Rx, it is commonly present within the subclone and can be used to estimate the size of the H30-Rx population. Additional analysis of the ST131 phylogenetic tree indicated two other isolates (DRR061436, DRR061456) should be assigned to H30-Rx, due to their close similarity with adjoining H30-Rx isolates, despite not exhibiting CTX-M-15 genes. Furthermore, we accounted for CTX-M-15 being present in non-H30-Rx subclonal populations. Despite exhibiting CTX-M-15 presence DRR061483 was deemed as non-H30-Rx due to its phylogeny, resulting in a total number of 27 H30-Rx isolates (69% of ST131 isolates). The results agreed with those of Kanamori *et al.* [67]. This dominance of H30-Rx within ST131 is likely due to its more extensive resistance profile compared with other ST131 isolates, even including the FQ-resistance associated with the H30-R subclone.

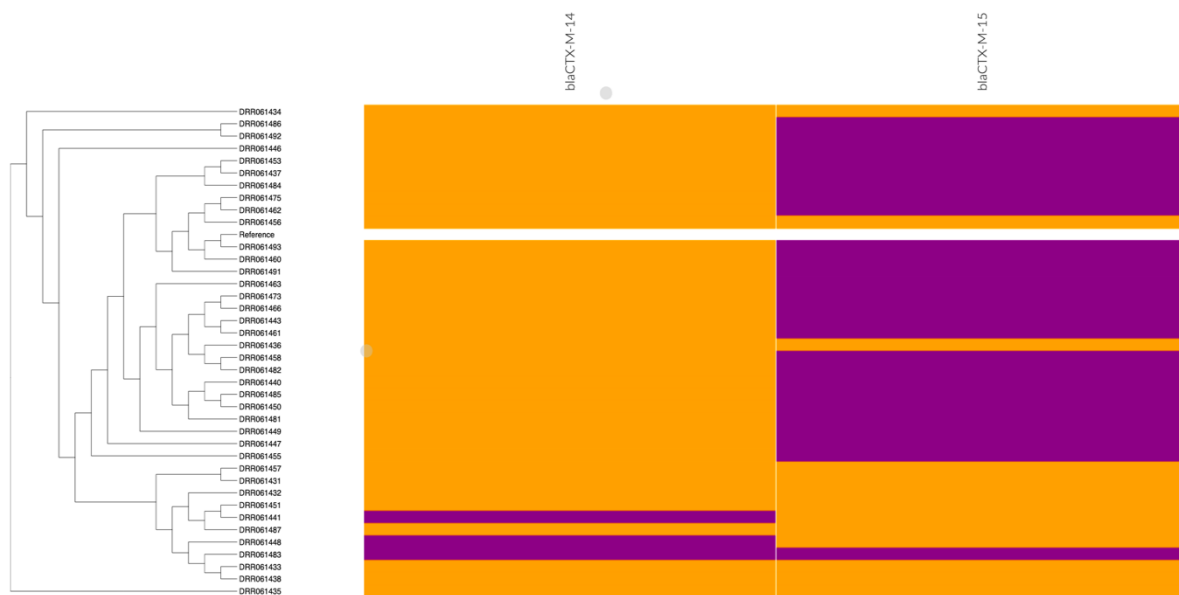


Figure 3.8: Presence of CTX-M-14 and CTX-M-15 ESBLs in ST131 *E. coli* isolates, obtained using abricate, where orange stands for gene absence and purple for gene presence.

CTX-M-15 was also the most common ESBL type in non-ST131 isolates (50%). Other CTX-M types were rare and mostly observed in non-H30-Rx ST131 subclones. The results obtained show the high incidence of CTX-M ESBLs within the analysed *E. coli* population, with the majority of isolates analysed containing at least one CTX-M. Out of the other ESBL types TEM-1 was most common (seen in 23 out of the 63 isolates analysed).

3.3.2.2 Resistance genes

The quinolone resistance of ESBL *E. coli* varies depending on their genetic profile. Quinolone resistance has been shown to originate from either *qnr* genes, encoding proteins capable of blocking ciprofloxacin action, or *aac(6')-Ib-cr* genes encoding an aminoglycoside acetyltransferase that confers reduced susceptibility to ciprofloxacin by its *N*-acetylation [97]. *QnrS1* was detected in only one isolate showing the predominance of *aac(6')-Ib-cr*, whose dramatic increase has been reported in the United States across recent years [97]. 8 out of 24 non-ST131 isolates (33%) contained *aac(6')-Ib-cr*, compared to 16 out of 39 the ST131 isolates (41%). Within ST131 15 of the 27 H30Rx (56%) isolates showed *aac(6')-Ib-cr* presence. This leads to show H30-Rx is associated with the highest percentage of quinolone resistance genes.

Next we evaluated the resistance to aminoglycosides, often associated with ESBL presence [98]. Aminoglycoside resistance has been shown to originate from the *aadA* gene group, capable of streptomycin and spectinomycin inactivation [99]. Within the analysed population ST131 isolates showed a higher frequency of *aadA* genes (10% *aadA1*, 72% *aadA5*) compared to non-ST131 (0% *aadA1*, 54% *aadA5*). The high resistance profile of H30-Rx was once again visible, as it exhibited the highest overall *aadA* gene percentage (15% *aadA1*, 77% *aadA5*). Furthermore, tetracycline resistance, conferred through the *tet* gene group [100], was visible in the majority of ST131 isolates (59% *tet(A)*) and more than 30% of non-ST131 (33% *tet(A)*, 38% *tet(B)*). Lastly, resistance to trimethoprim-sulfamethoxazole, conferred through the *sul* and *dfr* genes [101], was widely present within the whole analysed population with *sul1* (67% non-ST131, 82% ST131), *sul2* (63% non-ST131, 31% ST131) and *dfrA17* (58% non-ST131, 77% ST131) being most common. Once again resistance was most wide-spread among the H30-Rx population, with more than 80% isolates showing the presence of *sul1* and *dfrA17*.

H30-Rx isolates were shown to poses a significantly higher frequency of resistance genes than non-H30-R, making them significantly more likely to survive antibiotic treatments. Further analysis conducted by Kanamori *et al.* showed H30-Rx isolates were significantly enriched with specific virulence genes [67]. The high virulence and resistance capabilities of H30-Rx provide this subclone with a significant advantage over other ESBL producing strains contributing to its epidemiological success and prevalence observed within this study.

3.4 Conclusions

We have performed the genome analysis of 63 *E. coli* isolates, collected over 6 years in community hospitals in North Carolina for a study conducted by Kanamori *et al.* in 2017 [67]. ST131 was found to be the dominant lineage and mostly constituted of the highly resistant H30-Rx strain. We have shown CTX-M-15 is the dominant ESBL type in both ST131 and non-ST131 strains, with only a few other CTX-Ms detected in both populations. On average the ST131 clone group showed a higher resistance profile to antibiotic treatments than non-ST131 isolates. The H30-Rx subclone, showed the highest overall percentage of resistance genes making it the most MDR subclone within the analysed samples. The high resistance profiles observed explain the highly successful dissemination of ST131 and specifically H30-Rx in the recent years. The results obtained fully agree with the data reported by Kanamori *et al.*. Furthermore, the results obtained correspond with other reports focusing on *E. coli* infections in North America. In 2014 Chen *et al.* reported similar trends in clinical and community settings in North Carolina and Virginia, showing CTX-M-15 and CTX-M-14 producing ST131 as the dominant lineages [94]. Johnson *et al.* reported the H30 subclone as a leading cause of MDR *E. coli* infections in the United States in the years 2011-2012. The group analysed 360 isolates and H30 accounted for 83% of the analysed samples [102]. Lastly back in 2010 Peirano *et al.* associated high prevalence of ST131 with urinary tract infections in the Chicago area [103]. Our findings, associated with those of Kanamori *et al.*, show ST131 and its subclone H30-Rx are likely to become increasingly prevalent. New treatment methods must be developed in response to this dramatic raise in MDR *E. coli* populations. Furthermore, our study highlights the importance of surveillance of the *E. coli* populations world-wide to assess resistance transmission and equip clinicians with an understanding of current resistance profiles.

4. Literature review of antimicrobial therapies incorporating polymeric nanoparticles

Supervisor: Professor Alexander Cameron, University of Nottingham

Abstract

To effectively account for antibiotic resistance spreading new therapies must be developed to replenish the drug pipeline used against antimicrobials. The use of nanoparticle drug carriers is particularly promising within this field, due to their ability to eradicate bacteria, either through their native antimicrobial properties or by acting as carriers for antimicrobial drugs. Polymeric nanoparticles (PNPs) are of particular interest due to their ease of synthesis, low cost, biocompatibility, biodegradability, low toxicity, and high water solubility. In the following review we have analysed the current advances in PNP antimicrobial therapies, focusing on PNP materials of both natural and synthetic origin. The review was followed by an analysis of potential ways to incorporate this topic within a PhD project.

4.1 Introduction

The increasing resistance of bacteria to antibiotics and disinfectants, demonstrates an urgent need for novel treatments [54]. While preventative measures including infection control, antibiotic stewardship and new antibiotic development, can delay the spread of resistance, the dramatic genetic plasticity of bacteria makes wide-spread antibiotic resistance inevitable [111]. To prepare for this future, current antimicrobial approaches must be complemented by novel methods of tackling bacterial infections. A promising, alternative approach is the incorporation of nanoparticle (NP) drug delivery systems to selectively target and eliminate bacterial infections.

NPs are colloidal particles with diameters ranging from 1–1000 nm [112]. These low dimensions result in particular physical and chemical properties, considerably different from those of bulk materials [113]. The exciting aspect of NP treatments, is their ability to not only attack the bacteria themselves, but also act as carriers for antimicrobial compounds [113]. This approach has a significant advantage over the administration of nonformulated and free drugs. The incorporation of NP drug carriers can be implemented to reach challenging areas of the human body, such as the brain, facilitating the difficult transfer of antibiotics across the blood brain

barrier. Furthermore NP carriers can be incorporated to transport molecules of otherwise unfavourable pharmacokinetic profiles, including low aqueous solubility and high toxicity, enabling the safe and efficient use of these molecules [114]. Furthermore, the incorporation of drugs into NPs enables their prolonged systemic circulation and can lead to a reduction of treatment side effects [115]. Formulations containing NPs can be used to overcome common antibiotic resistance mechanisms, such as enzyme inactivation, decrease in cell permeability, overexpression of efflux pumps and modification of the target site [116]. This capability of enhancing the pharmacokinetic profiles and activity of drugs, places NP delivery systems at the forefront of today's research [115].

Areas where NP approaches hold significant potential to improve upon current antimicrobial treatments include; targeted, environmentally responsive antibiotic delivery, combinatorial antibiotic delivery, nanoparticle-enabled vaccination, and nanoparticle-based bacterial detection [117]. As of 2017 there were 15 antimicrobial NP formulations under clinical development, showing the use of NP in antimicrobial treatments is a rapidly developing area [118]. However, a number of issues regarding the safe use of NP delivery platforms still must be addressed. These include the biological interaction of NPs in patients and commercialisation hurdles related to manufacturing costs, and regulatory standards. Future NP delivery platforms should entail a minimum complexity in their design and development, to ensure their reproducibility and minimise toxicity [119].

Several groups of NPs are currently being utilised and studied. These include; polymeric nanoparticles, lipid-based carriers, dendrimers, carbon nanotubes, and silver or gold nanoparticles [120]. Within this group polymeric nanoparticles (PNPs) are of particular interest for several reasons including; ease of synthesis, low cost, biocompatibility, biodegradability, low toxicity, and high water solubility [120]. The following review focuses on PNP applications in antimicrobial treatments, followed by a discussion about future PhD research prospects within this area. As a plethora of information and publications is available, within this diverse field, this review focuses only on PNP materials, either exhibiting antimicrobial activity or being used as carriers for antimicrobial compounds.

4.2 Polymer nanoparticles

The particle size and particle size distribution of PNPs are of particular importance, as they define the materials properties and can be optimised depending on the mode of preparation [121]. For systemic injection NP sizes larger than 300 nm should be avoided, due to issues such as thrombosis and rapid elimination from the body through filter organs, namely the liver and spleen. Carriers smaller than 10 nm are directly excreted by the kidneys and therefore should also be avoided, resulting in the optimum PNP size ranging from 30 to 300 nm, with the possibility of being more finely tuned if required [122]. Size requirements for PNPs used for local treatments may be more specific. The size of PNPs used in ocular delivery must be small enough to allow permeation through the ocular barriers. Particle sizes of less than 200 nm have been advised for the treatment of the anterior eye region and sizes lower than 350 nm have been shown to be effective in treating infections in the posterior region [123]. In topical PNP formulations transport across the skin membrane was shown to increase with decreasing nanocarrier size, resulting in particles as small as 20 nm being favourable for such treatments [124]. The above examples show the site of action and mode of delivery must be considered when developing PNP formulations.

PNPs can be assembled from either pre-made polymers, or by direct monomer polymerization (Figure 4.1).

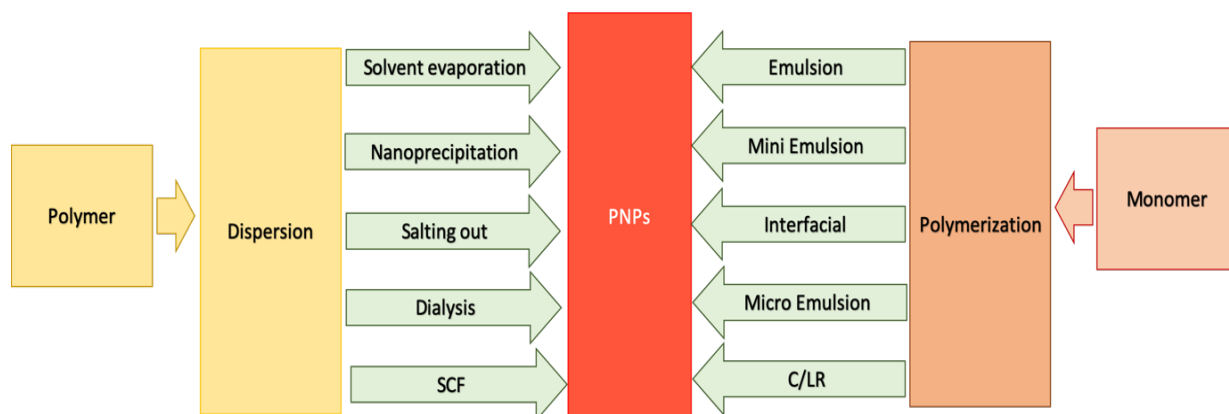


Figure 4.1: PNP preparation techniques, where SCF: supercritical fluid technology, C/LR: controlled/living radical [121].

Polymers used to construct PNPs can be lipophilic, hydrophilic or amphiphilic, enabling a wide range of compounds to be dispersed, dissolved, or chemically bound to the polymer chains [125]. This diverse range of polymer scaffolds can be engineered to respond to external triggers

such as pH ^{[126],[127]}, light ^[128], temperature ^[129] and enzymes ^[130], enabling a stimulus initiated, targeted drug release.

PNPs can be of synthetic or natural origin (polysaccharide and protein based) ^[131]. Natural polymers are easily metabolised by the body, thus have the advantage of excellent biocompatibility, however their use requires extensive purification to avoid an immunogenic response ^[131]. Synthetic polymers can be engineered with properties facilitating efficient target site delivery; such as charge, hydrophobicity and degradation profile and have the benefit of a higher purity and reproducibility than natural ones ^[132]. However, any synthetic polymer selected to synthesize PNPs, used for intravenous injection, must show good biocompatibility and biodegradability, to reduce toxicity and prevent polymer accumulation in the cells after repeated administration ^[120]. A series of guidelines should be followed when developing PNP formulations. Firstly, the drug needs to be effectively loaded onto the PNP and retained within the nanocarrier throughout the storage time. Secondly the PNPs need to be biocompatible, non-toxic and should not exhibit an immune response, to avoid inflammation and maximise the residence time within the patient's body enabling a sustained and effective drug release. Lastly the PNPs should selectively accumulate at the target cells, release the encapsulated drug and then become fully eliminated ^[122].

4.3 Polymer nanoparticles with antimicrobial properties

The antimicrobial activity of PNPs can originate from either their native properties or be a result of chemical modifications, such as conjugation with active molecules. As most bacterial cell walls are negatively charged under physiological conditions, the majority of antimicrobial polymers are positively charged. The electrostatic attraction between the cationic PNP and the cell leads to a destruction of the cell wall and/or the cytoplasmic membrane ^[133]. The simpler wall structure of Gram-positive bacteria enables a facile entry of molecules, compared to the more dense and complicated structure seen in Gram-negative bacteria, protecting them to a higher degree against the biocide ^[134].

4.3.1 Cationic antimicrobial PNPs

As antimicrobial activity is linked to the cationic character of the polymer, quaternary amines and phosphates are often incorporated within the scaffold [133]. Beyth *et al.* synthesised alkylated polyethyleneimine (PEI)-based nanoparticles containing quaternary ammonium salts within their scaffolds [135]. This material was then incorporated into dental resin and showed strong activity against the oral pathogen *S. mutans*, without leaching of nanoparticles, nor the loss of mechanical properties being reported. However, a major risk associated with the usage of these cationic compounds is haemolysis [136]. Nederberg *et al.* offered a solution to this issue, by synthesising biodegradable, triblock, cyclic carbonate PNP micelles, with the central block quaternized with triethylamine (**Figure 4.2**) [137]. The particles were shown to damage the cell wall and membrane of a range of Gram-positive bacteria (MRSA, *E. faecalis*, *C. neoformans*). Furthermore, the haemolysis of mouse red blood cells was evaluated, after incubation with the polymer, with no cell membrane damage being observed.

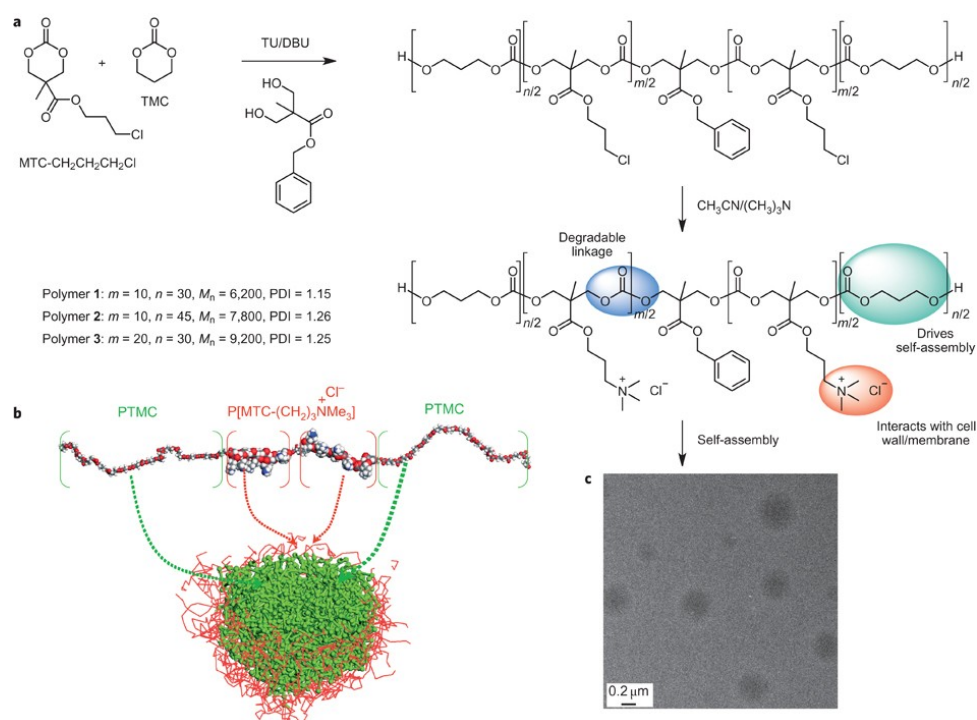


Figure 4.2: Structure of PNP scaffolds synthesised by Nederberg *et al.* **a)** synthesis pathway to obtain the amphiphilic co-polymers, **b)** PNP micelle formation **c)** TEM image.

Image taken from [137].

Alternatively, different scaffolds can be applied. Poly[2-(tert-butylaminoethyl) methacrylate] (PTA), has been reported to display high antimicrobial activity and low toxicity in humans [138]. PTA exhibits antimicrobial activity without quaternization, through the protonation of its secondary amine, with its mode of action based on the displacement of Ca^{2+} and Mg^{2+} ions within the outer bacterial membrane, followed by the membrane disorganization and the subsequent cell lysis [136]. Yuan *et al.* incorporated PTA-based ABC triblock copolymers into water-dispersible and biodegradable polymer micelles of good antimicrobial activity against *E. coli* and *S. aureus* (**Figure 4.3**) [139]. Song *et al.* included PTA in copolymer nanoparticles with ethylene glycol dimethacrylate. The copolymer was then used to functionalise silica nanoparticles. The silica-polymer conjugate showed an enhanced activity against both *E. coli* and *S. aureus*, compared to the free cationic polymer, with the antimicrobial activity increasing with decreasing size of nanoparticle [140].

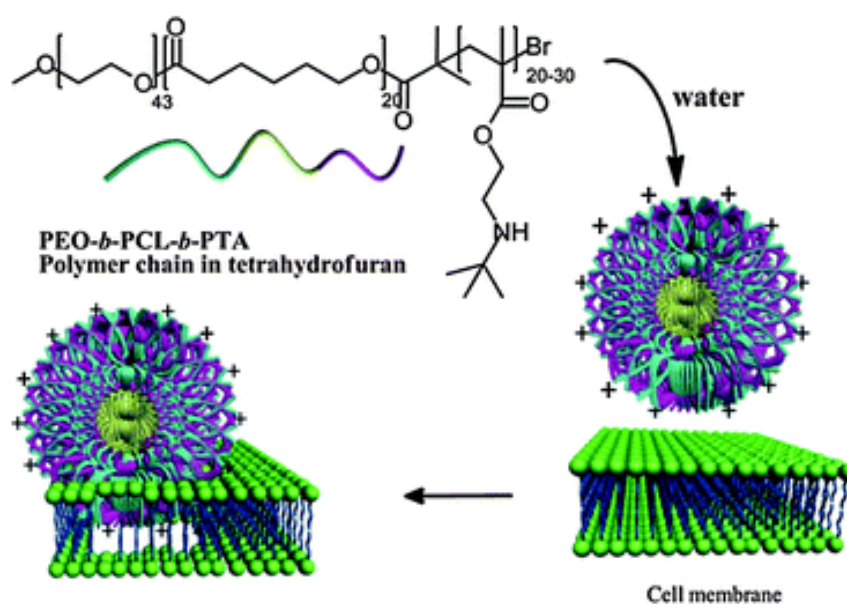


Figure 4.3: The self-assembly and action mechanisms of the PTA-based co-polymer synthesised by Yuan *et al.*. Image taken from [139].

4.3.2 Antimicrobial activity through scaffold functionalization

Polymers can be equipped with antimicrobial activity through the incorporation of cationic and hydrophobic functional groups within the polymer chain [141]. Ferguson *et al.* developed a library of dextrin-colistin conjugates exhibiting high activity against a range of Gram-negative pathogens and an improved toxicity profile and half-life compared to free colistin sulfate [142]. Furthermore, Tuross *et al.* reported the conjugation of polyacrylate nanoparticles with *N*-

thiolated β -lactams. These formulations were shown to exhibit high *in vitro* activity against MRSA and showed improved pharmacokinetic properties of the antibiotic, such as enhanced solubility [143]. Lastly functionalisation of inorganic nanoparticles with polymers has also been applied. Dong *et al.* functionalised magnetite nanoparticles with 2- (dimethylamino)ethyl methacrylate, quaternized with ethyl bromide and showed their high biocidal activity against *E. coli*. An advantage of this approach is the reusability of the nanoparticle, capable of being attracted to the container wall after pathogen eradication [144].

4.3.3 Antimicrobial PNPs of natural origin

Within the field of naturally occurring antimicrobial polymers, the polysaccharide chitosan, has been the focus of many research efforts [145]. Chitosan has been extensively studied, due to its good biocompatibility, facile administration and ease of modification. Compound properties including molecular weight (MW), deacetylation degree and positive charge, allow the alteration of its antimicrobial activity and solubility in aqueous media [146],[147]. De Paz *et al.* analysed the antimicrobial effect of different molecular weight (MW) chitosans, at natural pH, showing low-MW scaffolds exhibit an improved activity towards *S. mutans* biofilms [148]. The effect of pH on chitosan activity, against *E. coli* and *S. aureus* cultures, was further studied by Chang *et al.* The group reported that at acidic pH (pH 5.0 and 6.0), activity increased as the MW increased. By contrast, at pH 7.0, the chitosans with larger MWs significantly lost their activity, whereas the activities of the smaller MW chitosans increased. It was postulated this correlation results from variations in water solubility and zeta potentials of chitosans with various MWs, thus showing larger chitosans lose their activity at pH 7.0, correlating with the data previously observed by de Paz *et al.* [146], [148]. Additionally, the antimicrobial activity of chitosan can be further enhanced through conjugation with cationic molecules. A study by Tamara *et al.* showed an improved antimicrobial activity of chitosan-protamine conjugates, against *Bacillus cereus* and *E. coli*, compared to free chitosan. Moreover the conjugate did not appear to invoke a resistance response within the bacterium [145].

4.4 Polymer nanoparticles as carriers for antimicrobial drugs

Significant advantages associated with the application of PNPs in antibiotic delivery include high stability, high carrier capacity, possibility of incorporation of both hydrophilic and hydrophobic substances, and capability of variable routes of administration, including oral application and inhalation [149].

4.4.1 Micelles

Most commonly PNP carriers are obtained through a self-assembly process using amphiphilic polymers, capable of incorporating into micelle-like nanocarriers. The micelles hydrophobic core creates a microenvironment for the incorporation of lipophilic compounds, while the hydrophilic shell provides a stabilizing interface between the lipophilic core and aqueous surroundings, by that enhancing the colloidal stability and inhibiting aggregation and unwanted interactions with other components [150].

4.4.1.1 Natural polymers

A range of natural polymers has been used to encapsulate and deliver antibacterials. Costa *et al.* incorporated chitosan-coated alginate nanoparticles in the delivery of daptomycin across ocular epithelia. *In vitro* testing of ocular permeability showed an improvement in drug permeation, compared to free daptomycin. Furthermore, the antimicrobial activity of daptomycin was preserved after PNP encapsulation [151]. Piras *et al.* reported the use of chitosan nanoparticles to encapsulate an antimicrobial peptide (AMP) temporin B [152]. AMPs are a promising alternative to antibiotics, due to their wide-spectrum activity, rapid mode of action and low tendency to induce bacterial resistance [153]. Nevertheless, despite the initial enthusiasm associated with AMP applications their clinical use remains limited, due to poor stability in biological fluids, undesirable interactions with host macromolecules, and potential toxicity issues [152]. Piras *et al.* reported both the chitosan carrier particles, and the encapsulated AMP, exhibited a strong activity towards *S. epidermidis*. Furthermore, the study demonstrated a significant reduction in the toxicity of temporin B, showing promise for future use of AMPs in antimicrobial therapies [152].

4.4.1.2 Synthetic polymers

Within the synthetic polymer category, the most often employed materials include polyesters, polycarbonates, polyamides, and polypeptides [150]. Within this group poly(lactic-co-glycolic acid) (PLGA) or poly(ϵ -caprolactone) (PCL) exhibit excellent biocompatibility and biodegradability [115]. Han *et al.* incorporated PLGA nanoparticles in the treatment of *P. aeruginosa* biofilms. The nanocarrier was loaded with pyruvate dehydrogenase, an enzyme used to prevent biofilm assembly, that in its free form is unstable to denaturation under various storage conditions. The formulation was shown to disperse bacterial biofilms and exhibited activity for an extended period of time, compared to the free enzyme [154]. Shaaban *et al.* loaded both PCL and PLGA with imipenem and evaluated the formulations antimicrobial activity on *K. pneumoniae* and *P. aeruginosa* clinical isolates. The results showed an enhancement of imipenem activity and elevated the speed of bacterial eradication. Furthermore, the imipenem loaded PLC was reported to prevent biofilm assembly [155]. Despite their excellent properties PLGA and PCL are just a small fraction within the diverse library of materials that can be used to encapsulate drug molecules. For example, Liu *et al.* functionalised cholesterol-conjugated poly(ethylene glycol) with a transcriptional activator TAT peptide and used the resulting PNP to encapsulate antibiotics and transport them across the blood-brain barrier. The PNP lipophilic, cholesterol core enabled antibiotic encapsulation, while the hydrophilic TAT functionalised outside shell facilitated particle uptake by the brain. A rat model was employed to verify PNP presence within the brain showing effective antibiotic delivery to the site of action [156].

4.4.2 Polymersomes

Polymersomes are artificial vesicles made of amphiphilic copolymers, mimicking a cell membrane structure. The bilayer structure of the polymersome enables the encapsulation of both hydrophobic (inside the bilayer) and hydrophilic (in the interior) molecules [157]. Due to their amphiphilic character polymersomes are often compared to liposomes, however the polymeric vehicles show an improved stability through their higher molecular weight [158]. Hong *et al.* reported the high antibacterial activity of polymersome-hydrogel composites loaded with penicillin. The polymersome was assembled from poly(ϵ -caprolactone)-*block*-poly(lysine-*stat*-phenylalanine) and was shown to enable a sustained release of the antibiotic *in vitro*. Furthermore low cytotoxicity was reported, displaying the high potential of this

delivery platform (**Figure 4.4**) [159]. The wide range of polymers that can be incorporated within the copolymer, facilitates controlled and targeted release, however very little research within this field focuses on antimicrobial treatments, with cancer therapy being the principle focus [160][161]. Wayakanon *et al.* reported the use of polymersomes in the treatment of oral epithelial cells infected with *P. gingivalis*. The group incorporated a copolymer consisting of poly[2-(methacryloyloxy)ethyl phosphorylcholine] and poly[2-(diisopropylamino)ethyl methacrylate] to enable pH-controlled delivery of drugs to tackle intracellular-dwelling bacteria [162].

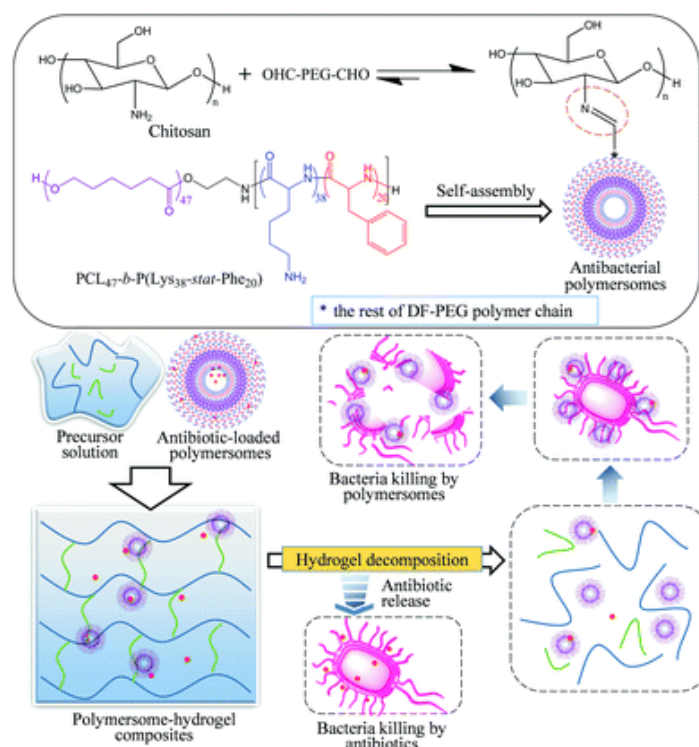


Figure 4.4: Graphical representation of the antibiotic-loaded polymersome-hydrogel composites synthesised by Han *et al.*. Image taken from [159].

4.4.3 Use of PNPs for targeted drug delivery

Local administration of antibiotics faces varying transport barriers unique to different organs, tissues, and subcellular compartments, as well as their pathophysiological states, which often prevent effective drug delivery. This includes; varying flow conditions and correlated sheer forces in different organs; ineffective permeation across various tissue types; and the ability of some bacteria to invade the immune response and survive inside host cells [163]. The high diversity of PNP scaffolds and conformations facilitates their programming with environmentally responsive switches, enabling tackling many of the above issues.

To overcome the challenges associated with high shear forces seen in organs, such as the heart or the lumen of the urinary tract, Zhang *et al.* placed PLGA nanoparticles loaded with ciprofloxacin in hydrogels showing strong adhesive properties. The nanoparticle-hydrogel hybrid exhibited superior adhesion and antibiotic retention under high shear forces when tested on mouse skin tissue [164]. Radovic-Moreno *et al.* targeted bacteria in acidic body environments through the synthesis of pH-responsive, surface charge-switching poly(d,l-lactic-co-glycolic acid)-*b*-poly(l-histidine)-*b*-poly(ethylene glycol) PNPs (**Figure 4.5**). The incorporation of a histidine residue within the scaffold enabled compound protonation in acidic conditions, such as present in the stomach, vagina, or bladder. The protonated histidine induced an overall positive zeta potential on the PNP surface, facilitating interactions with the negatively charged bacterial cell wall and producing a strong binding interaction. Once loaded with vancomycin the nanocarriers showed an improved activity of the antibiotic towards *S. aureus* [165].

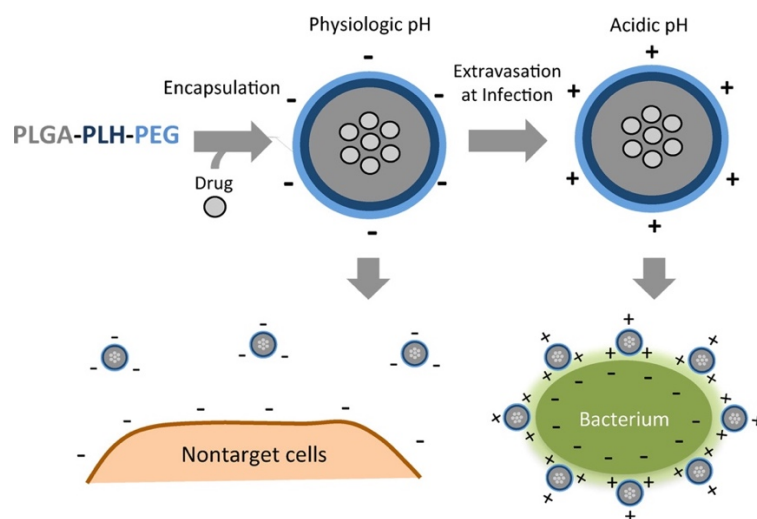


Figure 4.5: Schematic representation of the PNP designed by Radovic-Moreno *et al.*, the weakly acidic pH at the site of the bacterial infection induces the surface charge-switching of the PNP and drug release. Image taken from [165].

Insua *et al.* developed enzyme-responsive polyion complexes activated by the LasB elastase present in *P. aeruginosa*. This modification made the PNPs highly specific towards *P. aeruginosa* by that minimising antibiotic effect on commensal bacteria and toxicity towards human cells (**Figure 4.6**) [166].

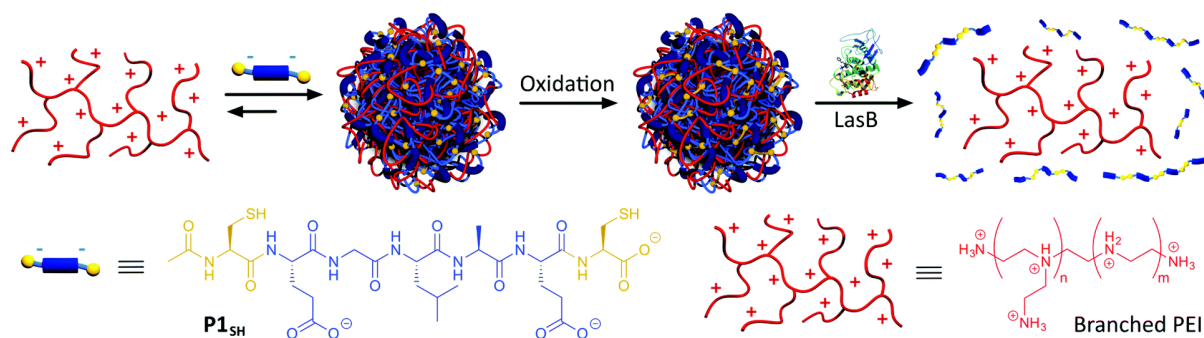


Figure 4.6: Assembly and oxidative cross-linking of the PNPs synthesised by Insua *et al.*, followed by enzymatic degradation and drug release. Image taken from [166].

Furthermore, a significant issue in treating bacterial infections is their ability to survive within epithelial cells and macrophages, leading to recurrent infections. Kisich *et al.* developed a system where moxifloxacin was encapsulated in poly(butyl cyanoacrylate) nanoparticles and was then taken up by macrophages infected with *M. tuberculosis*. The phagocytic nature of macrophages enhances their ability to accumulate nanoparticles and facilitates an easy delivery system to treat intercellular infections [167].

Additionally, PNPs can be employed to deliver combination therapies, involving the administration of the antibiotic, with an adjuvant that does not affect pathways necessary for bacterial survival, reducing the risk of resistance development. Quorum sensing (QS) is an attractive target, as while it enables the bacteria to communicate and form into a mature biofilm, its inhibition does not invoke a resistance response [168]. Singh *et al.* developed a formulation derived from modified alginate nanoparticles and used it to encapsulate the antibiotic ciprofloxacin and a QS inhibitor. The PNPs were equipped with a pH responsive linker, enabling a targeted drug delivery to the low pH of a *P. aeruginosa* biofilm. The combination therapy was shown to significantly reduce the viability of the biofilm, compared with ciprofloxacin treatment alone. Furthermore, the charge on the PNP and the release of a QS inhibitor led to a deep penetration of the carrier within the biofilm [169]. Alternatively, therapies including multiple active ingredients can be employed. Pandey *et al.* encapsulated three antitubercular drugs: rifampicin, isoniazid and pyrazinamide, into poly(D,L-lactide-co-glycolide). The administration of the conjugate to guinea pigs every 10 days, resulted in complete sterilisation of the lungs after only five doses compared to 46 oral daily doses of the free drug necessary to achieve an equivalent result [170].

4.5 Conclusions and PhD prospects

The field of polymer nanoparticles is highly diverse and rapidly developing. A plethora of materials and approaches can be undertaken to treat bacterial infections, providing researchers with a wide range of opportunities. A PhD within this field could either focus on the exploration of new materials and their antimicrobial and drug delivery capabilities or focus on the delivery of novel treatments and compound combinations, not explored previously.

In terms of novel polymeric scaffolds an area of potential exploration could be the incorporation of azobenzene moiety within the PNP (**Figure 4.7**). Azobenzenes capability to undergo a conformational change between its *trans*- and *cis*- isomers, upon light irradiation, has been under extensive study due to its wide application range [171], [172], [173], [174]. The conformational change leads to a significant structural modification. Within the *trans*- isomer the two aromatic rings are planar, while the *cis*- isomerisation pulls them close together and tilts them. This results in a change in substituent distance, with the substituents in the *para* positions of the aryl groups shifting from a distance of approximately 13 Å in the *trans* isomer, to 5–9 Å in the *cis* isomer [175]. This dramatic change presents a range of opportunities from a chemical perspective.

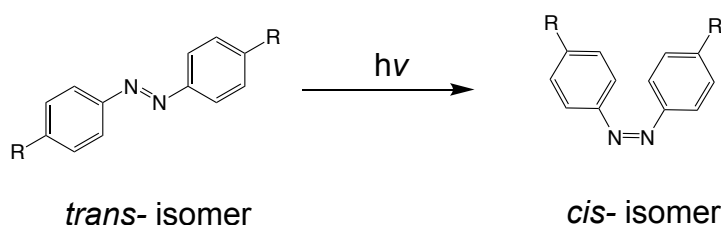


Figure 4.7: Azobenzene isomer structures.

Azobenzene derivatives have been shown to enable reversible photocontrol of the function of nucleic acids, peptides, enzymes, receptors, and ion channels in cells and in animals [175]. Furthermore, they can also be applied in the targeted delivery of antimicrobials. The application of light as the activating agent is favourable, as in principle it is not harmful and can be easily and precisely delivered. However, UV light is known to be toxic to cells and should be avoided as the activation wavelength. For that reason an effective photopharmacological agent should ideally be responsive to light between 650 and 900 nm [176]. Wegener *et al.* reported chemical modifications of an azobenzene photoswitch moiety,

attached to a diaminopyrimidine scaffold, with the aim of modifying their activation wavelength from UV to visible light. The modification of the aryl substituents enabled the control of the compounds activity against *E. coli* in both directions, using light in the visible region [177]. This potential for structure and activity diversity makes azobenzenes excellent candidates for activity switches. However, despite the depth of research conducted on azobenzenes very little has been done regarding their incorporation into PNPs and subsequent antimicrobial treatment. Kehe *et al.* demonstrated a dramatic reduction in bacterial biofilm formation by utilising an azobenzene coating. A continuous exposure of this surface to light resulted in its photofluidization (softening of the surface) caused by the rapid *trans*–*cis*–*trans* isomerization cycles. This property was then used to disrupt biofilm formation of *P. aeruginosa* and was reported unlikely to invoke resistance mechanisms [178]. Yao *et al.* incorporated azobenzene scaffolds into liposomes to enable light-controlled drug release in cancer treatments. The simultaneous use of UV and visible light facilitated the reversible photoisomerization of azobenzene and a controlled release of the chemotherapeutic drug doxorubicin [179]. Furthermore, Tamesue *et al.* developed photoswitchable supermolecular hydrogels consisting of cyclodextrins and azobenzene. Light activation enabled the control of the hydrogel properties through its morphology [180].

In addition to light activation, the release of azoreductase enzymes by some bacteria has been shown to selectively cleave azo bonds, facilitating a further mode of targeted delivery [181]. Azo bond containing compounds have been extensively studied for selective colonic delivery, through the use of prodrugs such as sulfasalazine, ipsalazine, balsalazine and olsalazine [182],[183]. The azo moiety is used to link the active molecule to the carrier scaffold. Following the prodrug delivery into the colon the azo bond is cleaved by azoreductases released by colonic bacteria, enabling a targeted drug delivery [183]. The use of azoreductase drug activation is not limited to the colon and has been widely applied in other fields, demonstrating it's a diverse and promising activation pathway [184],[181]. Additionally, azobenzenes have been reported to isomerise in response to pH changes providing further methods of controlled release, especially when targeting the acidic pH seen in biofilms [185],[186].

A range of materials could be prepared to incorporate the azobenzene scaffold. An example could be the use of poly- β -amino-esters (PBAE) (**Figure 4.8**). PBAEs are biodegradable and positively charged and obtained from the co-polymerization of diacrylate and amine molecules [187].

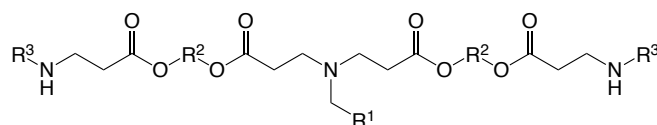


Figure 4.8: PBAE structure, where azobenzene scaffolds can be incorporated as group R².

In addition to their low cytotoxicity, PBAEs show promise in terms of acting as oligonucleotide carriers [188], [189], [190]. The use of antisense therapy incorporating short, single-stranded oligomers, complementary to RNA sequences within the target organisms, enables the silencing of genes and prevents unintended toxicity being invoked on commensal bacteria [191]. However, the application of antisense therapy has so far been challenging. Viral delivery has until recently been the most promising approach, however it has proven problematic due to cargo capacity, production and safety issues. The use of PBAEs provides a promising nonviral delivery alternative, due to their ability to effectively condense DNA, large potential for structure diversity, ability to buffer the endosome, and promote endosomal escape, low cytotoxicity compared to other cationic polymers and biodegradability via hydrolytic cleavage of ester groups [189], [190]. Furthermore, while antisense therapy has been widely explored when targeting eukaryotic cells, relatively little research has been done on prokaryotic gene inhibition making this an unexplored research area [192]. The research into PBAE-based approaches to deliver oligonucleotides to bacterial cells could provide vital information regarding the application of this field in antimicrobial treatments.

Additionally, PNPs could be used to deliver combination treatments. The incorporation of multiple mechanisms to fight bacteria has been proven to lower the risk of resistance development, with agents directed against a single target shown to be less successful than those directed against multiple molecular targets or against multisubunit macromolecular machines or structures [193]. As mentioned in this review Singh *et al.* showed excellent biofilm eradication through the use of antibiotics with QS inhibitors [169]. Biofilm formation has been shown to be a contributing factor in resistance observed in such problematic bacteria as *P. aeruginosa*, making QS inhibition an attractive target. As QS is not directly involved in the processes essential for bacterial growth, its inhibition does not impose harsh selective pressure for development of resistance, as seen with antibiotics [194]. Furthermore, a combination approach using both antibiotics and QS inhibitors has been shown to increase the susceptibility of bacterial biofilms to antibiotics *in vitro* and *in vivo* [195]. Despite these findings little combination therapies of antibiotics and QS inhibitors have been developed so far. While there

are multiple formulations in the clinic that combine antibiotics and β -lactamase inhibitors (eg. Tazocin, Unasyn, Augmentin) none so far have incorporated QS inhibitors to disrupt bacterial biofilms [¹⁶⁹]. This relatively unexplored field could be another topic undertaken in a potential PhD research project.

5. Conclusions

The rapid dissemination of antimicrobial resistance is one of the greatest challenges facing humanity, the importance of which will continue to grow as we run out of effective antimicrobials. This report examined different approaches to control and prevent the spread of antimicrobial resistance and highlights the dramatic pace at which resistance to drugs and disinfection methods develops. First, we have shown the current disinfection methods, applied in the clinic, may soon become insufficient when targeting *E. faecium* strains, due to the high adaptability and genetic plasticity of this pathogen. This was followed by the genomic analysis of MDR *E. coli* isolates obtained from hospitals in North Carolina by Kanamori *et al.* where we showed the high dissemination of the multidrug resistant clone ST131 and its subclone H30-Rx. Lastly, we have shown an interesting, new alternative to antimicrobial treatments, based on the use of nanoparticles to selectively target and eradicate bacteria. The use of polymeric drug delivery platforms has the potential to solve many of the issues associated with antimicrobial resistance; by enhancing the effect of old antibiotic treatments, enabling the use of drugs with poor pharmacokinetic profiles and selectively targeting the pathogen within the body. We have further analysed the prospects of a PhD research project within this field showing a wide range of available pathways and developments that could be undertaken.

6. References

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