

A research project report submitted by

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MRes. Antimicrobials and Antimicrobial Resistance (AAMR)

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Abstract

Antimicrobial resistance is rapidly becoming one of the greatest threats to modern healthcare across the globe. The aims of this work were to explore two distinct projects, to understand the variety of challenges we must overcome if we are to tackle the AMR crisis.

The first project focused on using mathematical modelling to better understand the interaction between *Pseudomonas aeruginosa* and its adhesion to human epidermal cells in an *in vitro* infection model. It was determined that the ability of *P. aeruginosa* to bind to human cells enables growth in higher concentrations of antibiotic than would otherwise be possible. This is due to the increased growth rate of *P. aeruginosa* when bound to the human epidermal cells. The model also suggested there were no differences in growth rate between meropenem-susceptible and resistant strains. These findings suggest that this type of resistance is likely to persist in populations. Additionally, it suggests that prevention of *P. aeruginosa* adhesion could be an effective method of preventing the spread of infection.

The second project aimed to investigate the presence of AMR in local human populations by analysing the influent of a wastewater treatment plant (WWTP). High levels of extended-spectrum beta-lactamase resistance were discovered. All but one *Enterobacteriaceae* isolated harboured the CTX-M beta-lactamase, with a considerable percentage of bacteria containing multiple *bla* genes. Sequencing of $bla_{CTX\text{-}M}$ genes showed the bacterial populations within the WWTP harbour similar *bla* genes to those found in bloodstream infections in the UK. This suggests that surveillance of WWTPs can be used as a proxy for surveillance of AMR within a local human population.

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Abbreviations

AMC	Amoxicillin-clavulanic acid
AMP	Ampicillin
AMR	Antimicrobial resistance
ATM	Aztreonam
AZM	Azithromycin
BLAST	Basic Local Alignment Search Tool
C	Chloramphenicol
CFQ	Cefquinome
CFU	Colony forming units
CIP	Ciprofloxacin
CLSI	Clinical & Laboratory Standards Institute
CPD	Cefpodoxime
CRE	Carbapenem-resistant *Enterobactericeae*
CTX	Cefotaxime
DMEM	Dulbecco's Modified Eagle's Medium
ESBL	Extended-spectrum beta-lactamase
F	Nitrofurantoin
FOX	Cefoxitin
HGT	Horizontal gene transfer
IPM	Imipenem
KPC	*Klebsiella pneumoniae* carbapenemase
MBL	Metallo-beta-lactamase
MDR	Multi drug-resistant

MRSA	Methicillin-resistant *Staphylococcus aureus*
NA	Nalidixic Acid
NCBI	National Centre for Biotechnology Information
OD	Optical density
ODE	Ordinary differential equation
OMP	Outer membrane protein
OT	Oxytetracycline
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
S10	Streptomycin
SXT	Sulfamethoxazole-trimethoprim
UTI	Urinary tract infection
WGS	Whole Genome Sequencing
WWTP	Wastewater Treatment Plant
XDR	Extensively drug-resistant

1. Current State of Antimicrobials and Antimicrobial Resistance

1.1. What are antimicrobials?

Antimicrobials is a term for a wide range of drugs used to treat infections caused by microorganisms, which can be grouped into various categories: antibiotics, antifungal, antiviral and antiparasitic drugs, which are used against bacterial, fungal, viral and parasitic infections respectively [1]. Antimicrobial resistance (AMR) occurs when some or all of the microorganisms causing an infection are able to survive treatment with these medicines. These surviving populations are then able to spread from person to person. Not only does this cause increasing levels of resistance in local settings such as hospitals or communities, but due to the nature of travel in the 21st century, it has the effect of spreading resistance around the globe [2]. This is further exacerbated by the ability of bacteria to transfer genetic material between populations, called horizontal gene transfer (HGT)[3], meaning that resistance can spread even in the absence of antimicrobial therapy.

The use of antimicrobials as we know them today began in the early 20th century with Paul Ehrlich and his "magic bullet." From his work using dyes to stain different types of tissue, he aimed to find compounds that could destroy microorganisms at dosage levels that would be safe for use in humans [4,5]. He systematically screened various arsenic derivatives based on the toxic drug Atoxyl, used for treatment of infections by trypanosomes at the time [5]. In 1909, the compound numbered 606 was found to be effective against syphilis in rabbits, and following trials in humans, went on to be marketed as the drug Salvarsan [6]. Modifying the chemical structure of Salvarsan led to the production of Neosalvarsan in 1912, a less toxic and more soluble version of the drug [7].

This approach to finding new drugs led to the discovery of sulphonamidochrysoidine, better known as Prontosil, by Gerhard Domagk. Screened using haemolytic streptococcus infected mice, it was discovered to

be an effective antimicrobial in 1932 [8]. Soon after, it was discovered that the drug was metabolised in the body, breaking down into the compound sulphanilamide, which retained all the antimicrobial properties of Prontosil [9,10]. Sulphanilamide was cheap to produce and unpatentable as it had been used for many years in the dye industry, which led to its widespread use as an antimicrobial as the first in the class of sulphonamides [6,9].

Meanwhile, penicillin had been discovered by Alexander Fleming since 1928, but he had failed to be able to purify it, and had given up the venture by 1940 [6,11]. Fortunately, in that same year, a team at Oxford led by Ernest Chain and Howard Florey published a paper on purifying penicillin in quantities high enough for clinical testing [12]. This breakthrough led to the mass production of penicillin in 1945, and eventually to the golden age of antibiotics [6,10].

1.2. Beta-lactams

Of all the antibiotic classes from the beginning of the antibiotic era to the modern day, by far the most widely used in the treatment of bacterial infections are the beta-lactams [13]. These are bactericidal compounds that act to inhibit bacterial cell wall synthesis. Inside a bacterial cell, specific enzymes are used to cross-link the multiple layers of peptidoglycan. These enzymes are the transpeptidases, transglycolases and carboxypeptidases otherwise known as penicillin binding proteins (PBPs) [14–16]. PBPs are found in the inner membrane, so the first step for the beta-lactams is to cross the outer membrane. This is achieved by passing through outer membrane proteins known as porins or OMPs, that form hydrophilic channels across the membrane [17].

The beta-lactams are structurally similar to the substrate of PBPs, and so act as mechanistic inhibitors by binding to these enzymes [13]. As cell wall synthesis is an ongoing process, parts of the cell wall are being broken down at the same time as new layers of peptidoglycan are being produced. Without new layers

being cross-linked due to the action of beta-lactams, the bacterial cell becomes structurally weakened and eventually bursts due to osmotic pressure [18].

Alexander Fleming's famous discovery of penicillin in 1928 led to mass production and use of this antibiotic in the 1940s [19]. Within a year, 4 strains of *Staphylococcus* were found in hospitalised patients that were resistant to penicillin [20] and by the end of the 1960s, resistance to penicillin was found in over 80% of staphylococcal isolates from both community-acquired and hospital-acquired infections [21].

In the decades that followed the production of penicillin, synthesised modifications to the side chain of the beta-lactam ring were used to develop various semi-synthetic penicillin derivatives [22]. Alongside this, widespread screening of environmental bacteria led to the discovery of entirely new classes of antibiotics, as well as many novel beta-lactams. These include the discovery of further types of beta-lactams such as the cephalosporins from *Cephalosporium acremonium* [23], as well as the discovery of monobactams from *Pseudomonas acidophilia, Chromobacterium violaceum* and *Agrobacterium radiobacter* [22].

The fact that cephalosporins were resistant to common penicillinases made them ideal for treatment of the most clinically relevant penicillinase-producing bacteria of the time, *Staphylococcus aureus.* They were also found to be effective against streptococcal infections as well as non-beta-lactamase-producing gram-negative bacteria [13]. As ever, resistance to cephalosporins quickly followed their introduction, with the TEM beta-lactamase being increasingly found on transmissible elements in gram-negative bacteria [24]. This led to the development of second and third-generation cephalosporins, that were stable against this enzyme, and with greater activity against the gram-negative bacteria likely to carry it [13].

As the number of clinically relevant beta-lactamases increased during the 1960s and 70s, the need for a way to combat these enzymes became ever more important [18,25]. Since the streptomycetes were (and still are) the largest known producer of natural antimicrobial compounds, vast amounts of the bacteria

were grown in fermentation broths and screened for the production of useful compounds [26].

In 1976, the compounds olivanic acid and clavulanic acid were isolated from *Streptomyces olivaceus* and *S. clavuligerus* respectively; these were the first beta-lactamase inhibitors [27]. Around the same time, a compound named thienamycin was isolated from *S. cattleya* during soil-screening for antibiotics that inhibit the synthesis of the bacterial cell wall. This was found to have a broad spectrum of antibiotic activity and was not inhibited by the presence of many beta-lactamases that usually conferred resistant to earlier penicillins and cephalosporins [28].

Thienamycin was found to be very chemically unstable in solution, and so could not be used in concentrations necessary to have an antimicrobial effect *in vivo* [18,29]. This led to the development of synthetic derivatives of thienamycin, firstly the N-formimidoyl derivative imipenem; later followed by meropenem, ertapenem and doripenem among others [18,30,31].

1.3. Resistance to beta-lactams

Although the first documented cases of resistance to beta-lactams were caused by beta-lactamases in *Staphylococcus*, this is not the most common method of resistance in gram-positive bacteria. The primary resistance mechanism for these bacteria is structural modification of the PBPs resulting in reduced affinity for beta-lactams [18,32]. An example is the case of *S. aureus*, where acquisition of a mobile element containing the transpeptidase PBP2a from another staphylococcal species produced methicillin-resistant *S. aureus* (MRSA) [33].

Among gram-negative bacteria there are a variety of resistance mechanisms due to the ability of the outer membrane to restrict entry of beta-lactams to the cell. To enable uptake of nutrients, the outer membrane contains porins, proteins which form water-filled channels across the membrane [34]. These provide entry to the cell to hydrophilic compounds of a certain size that varies

by organism. Resistance due to changes in these proteins can be achieved either by loss of porins altogether, decreased size, lower expression levels, or a combination of these mechanisms [35]. The porin OprD is the main channel used by carbapenems to pass through the outer membrane of *Pseudomonas aeruginosa* [36]; this has been well studied as an acquired mechanism of carbapenem resistance. Mutations in external loops of this protein lead to a decrease in size of the channel and reduced susceptibility to imipenem [37], similar to results of mutations in OmpC of *Escherichia coli* and other beta-lactams [38].

Efflux pumps are another well studied mechanism of beta-lactam resistance in *P. aeruginosa*. They are systems made up of a protein transporter, a periplasmic connective protein and an OMP; which use proton motive force to transport substances, including antibiotics, outside the cell [39]. Mutations in the regulatory proteins of these systems in *P. aeruginosa*, such as MexR in MexAB-OprM system, have shown an increased expression of efflux pumps and increased resistance to beta-lactams [40,41]. Efflux pump systems can be especially problematic because they can include multiple antibiotic classes in their substrates. When combined with resistance due to reduced outer membrane permeability this can lead to multidrug resistant (MDR) pathogens [39].

The primary resistance mechanism among gram-negative bacteria is the production of beta-lactamase enzymes [32]. Beta-lactamases are classified into groups either based on their structure, as in the Ambler classes A-D; or their substrate and inhibitor profiles as in the Bush-Jacoby classification [42,43]. In the latter, group 1 enzymes are cephalosporinases found in the Ambler class C. Group 2 are the serine beta-lactamases from Ambler groups A and D. This is the largest group of these enzymes, as it contains the ESBLs, including the TEM, SHV and CTX beta-lactamases. The group 3 enzymes consist of the metallo-beta-lactamases that comprise the Ambler group B. These are unique in their structure in that they need zinc ions at their active site instead of serine,

meaning they are unaffected by beta-lactamase inhibitors such as clavulanic acid or tazobactam [43].

Many species of bacteria harbour chromosomally-encoded inducible beta-lactamases, that are thought to be related to PBPs [44]. However, it is the transferable genetic elements that pose the biggest threat. Surveillance studies in the 1970s found that an average of 63% of all ampicillin-resistant bacteria contained one of either the TEM-1 or TEM-2 penicillinases [32]. Following this, many new beta-lactam antibiotics were developed that were specifically designed to be resistant to these enzymes. Unfortunately, with as little as 3 amino acid substitutions, the TEM-1 penicillinase is capable of hydrolysing these broad spectrum cephalosporins [45]. This new enzyme was named TEM-3, and was the first expanded-spectrum beta-lactamase (ESBL) to be discovered [44]. These are usually found on mobile genetic elements and are characterised by their ability to hydrolyse third and fourth-generation cephalosporins as well as monobactams [46].

Throughout the 1980s and 90s, the most common ESBLs were enzymes descended from the TEM and SHV penicillinases [32,44]. More recently, a new family of ESBLs has spread to become the dominant group across the globe. First discovered in 1989, the CTX-M beta-lactamases have now nearly displaced all other ESBLs among the *Enterobacteriaceae*, including the previously dominant TEM and SHV enzymes [46]. The most common of these new enzymes is CTX-M-15, in part because it is often carried on the particularly successful *E. coli* strain ST131 [46].

As rising resistance to penicillins led to a wider use of cephalosporins, so increased levels of cephalosporin resistance led to wider use of carbapenems [47]. As ever, the increase in clinical use has been swiftly followed by an increase in resistance [18]. Of the group 2 (Ambler class A) carbapenemases, the *Klebsiella pneumoniae* carbapenemase (KPC) enzymes are the most widespread. Since the first KPC was discovered in *K. pneumoniae* in the US in 1996, the enzyme has spread to a vast number of other species

including *E. coli*, *P. aeruginosa, Enterobacter spp*., *Acinetobacter spp*., *Salmonella enterica*, and *Serratia marcescens* [48,49].

KPC-producing bacteria have spread from the US to every other continent (bar Antarctica) on the planet. In the US the spread has been due to the expansion of a particular strain of *K. pneumoniae*, ST258; whereas in countries such as the UK the spread is due to plasmid expansion [49].

Of the other groups of carbapenemases the MBLs are the most clinically relevant because they do not physically bind to the beta-lactam ring as the serine-based enzymes do; therefore, they are not inhibited by commercially available beta-lactamase inhibitors such as clavulanic acid [50]. The first transferable MBL, IMP-1, was found in *P. aeruginosa* in 1988 in Japan [51]. Since then it has spread amongst the *Enterobacteriaceae* and *Ancinetobacter* spp. and across the globe to Europe, South America and the US [52].

In 2008 the first case of *K. pneumoniae* with New Delhi Metallo-beta-lactamase (NDM-1) was found in a Swedish patient who had returned there from India [53]. This is of particular concern because the plasmid on which this gene is transmitted also carries genes which confer resistance to aminoglycosides, macrolides, rifampicin, sulfamethoxazole aztreonam (a monobactam not usually hydrolysed by NDM) [50]. As of 2017, 17 variants of NDM have been isolated [54] and the spread of these genes can be seen all across the globe [50].

1.4. Containing the AMR crisis

The UN has called the AMR crisis a "global health emergency" [55], while the former WHO Director-General, Dr Margaret Chan, has referred to AMR as "a slow-motion tsunami" [56]. Globally, AMR is responsible for the deaths of at least 700,000 people per year [57], and it has been estimated that this figure could rise to 10 million by 2050, although this is disputed as too high an estimation [58,59]. Undoubtedly, if actions are not taken the AMR crisis will have a dramatic impact on medicine as we know it. Medical treatments such as routine surgery, joint-replacements and caesarean sections all rely on the efficacy of antibiotic

therapy. Treatment that requires immunosuppression such as chemotherapy and organ transplantation would be impossible if antibiotics were no longer effective, as indeed is already becoming a problem with the rise of MDR and XDR bacterial infections [60]. This potential future is what the WHO calls the "post-antibiotic era" [61], and we must act quickly and effectively to prevent it.

In January 2019, the UK government released its five-year national action plan on AMR [62]. This report mirrors some of the key areas outlined in the WHO and EU action plans, as well as the O'Neill report commission by the UK government in 2014 [58,63,64]. One of the main issues identified in these documents is the need for appropriate use of antimicrobials. Public Health England released a report that 20% of antibiotics prescribed in primary care in England are inappropriate [65], while the CDC estimates that over 30% of antibiotics prescribed in the US are unnecessary [66]. In low- and middle-income countries, antibiotics are often available without prescription, either due to lack of regulation, or lack of enforcement of existing regulations [67]. In some developing countries, available antimicrobials are often of poor quality, due to inadequate storage or even outright counterfeit drugs, which do not contain the active substance they are supposed to [68]. It is estimated that the level of antibiotics used in humans is comparable to that used in the agricultural industry [58], with the latter figure set to rise by 67% by 2030 [69]. Additionally, many of the antibiotics used are provided for growth promotion and the prevention of disease, rather than the treatment of infections [70]. It is documented that levels of resistance in a region are driven in large part by the types of antimicrobials used in that area [71], so the proper use of antibiotics is an important step in any attempt to prevent the increase in AMR.

Another of the key areas highlighted in the UK action plan is "investing in innovation, supply and access," which includes the production of new antimicrobials. A list of priority pathogens was published by the WHO, detailing those that should be the focus of research and development for new antimicrobial medicines [72]. It lists the carbapenem-resistant strains of *Acinetobacter baumanii*, *P. aeruginosa* and the *Enterobacteriaceae* as critical

priority pathogens; while the high priority pathogens include resistant strains of *Enterococcus faecium* and *S. aureus*, among others. The WHO report on antibacterial agents in clinical development found that there are 42 new compounds that target the high and critical priority pathogens, but only two of these show potential against all three priority organisms [73]. The need for broad-spectrum antibiotics is paramount, as treatment of severe infections generally cannot wait for the results of antibiotic susceptibility testing. Additionally, the need for therapeutics with new mechanisms of action is urgent, as bacteria will not have developed resistance mechanisms from related compounds [74].

The first project detailed below focused on forming a mathematical model of bacterial growth based on attachment experiments in *P. aeruginosa*. This was based on work by Roberts *et al.* that used modelling to explore novel therapeutic options for burn-wound infections in a rat model. The project looked at the growth rates and interaction between bacterial cells bound to human cells and those of the free-swimming population, as well as those of both meropenem-resistant and susceptible strains. The objective of the project was to create a model that could potentially offer insight into the mechanisms of AMR in these strains.

The second project focused on surveillance of AMR in bacterial populations isolated from separate inlet pipes to a local waste water treatment plant (WWTP). One objective was to look for at AMR in *Enterobacteriaceae* isolated from the two populations and determine any difference in the frequency of specific genes or sensitivity profiles. The project also aimed to discover if specific AMR genes found in clinical settings in the UK could be found in this WWTP, so attempts were made to detect ESBL and carbapenemase producing bacteria.

2. Mathematical modelling of *Pseudomonas aeruginosa* and human epidermal cells *in vitro*

2.1. Abstract

We develop a mathematical model to simulate the growth dynamics of *P. aeruginosa* adhesion *in vitro*, in the presence and absence of meropenem. Fitting this model to experimental data we obtain parameter values that give us insight into the mechanisms of antimicrobial resistance. Our results suggest that the ability of this bacteria to bind to host cells facilitates higher growth levels, and therefore a higher concentration of antibiotic is required to be effective in reducing population size. We also find no difference in growth rates between our meropenem resistant and susceptible strains, suggesting that the mechanism of carbapenem resistance in our *P. aeruginosa* strain may have negligible effect on bacterial fitness *in vivo* and so is likely to persist in populations.

2.2. Introduction

Antimicrobial resistance (AMR) is one of the most complex and challenging public health issues the world has ever faced [75]. Levels of AMR are increasing rapidly across the globe [76] and a case has been made that it will claim the lives of 10 million people a year by 2050 [58].

Ever since resistance has been an issue it has largely been found in a hospital setting [77]. One of the most severe and frequent causes of hospital acquired infections in the world is *P. aeruginosa* [78,79]. It is the most frequent cause of burn wound infections, as well as chronic respiratory infections in cystic fibrosis patients [80,81]. It is also commonly associated with ventilator associated pneumonia, community acquired pneumonia, urinary tract infections, bacteraemia and enterocolitis [78].

One of the major factors in *P. aeruginosa* pathogenicity is its ability to adhere to host cells during infection [82,83]. It is also intrinsically resistant to a wide range

of antibiotics [78,84,85], and the mechanisms of resistance are ever increasing, leading to the rise of multidrug-resistant (MDR) and extensively drug-resistant (XDR) infections [78]. Because of this, it is considered to be of critical priority as a target for new antimicrobial drugs by the WHO [86].

Unfortunately, at the same time as we are seeing the increase of AMR across the globe, the production of new antimicrobials is heading in the opposite direction [87]. As conventional approaches have not led to new drugs, we need a multidisciplinary approach [77,88]. Mathematical modelling is increasingly being used as a tool to help contend with the global AMR crisis. It has seen use in a variety of ways, such as modelling the transmission of AMR [89–94], specific mechanisms of AMR [95,96], as well as aiding in new drug discovery [97].

In this project, we develop a system of ordinary differential equations to describe the dynamics and treatment of *P. aeruginosa* in an *in vitro* adhesion model using both antibiotic susceptible and resistant populations. This is based upon the work of Roberts *et al.* [98] who used a system of ODEs to describe the behaviour and treatment options of an infected burn wound. We modify this system to include an antibiotic term similar to Spalding *et al.* [99] to complete our model.

2.3. Materials and Methods

2.3.1. Bacterial strains

The *P. aeruginosa* strains used were the meropenem susceptible PA1004 and meropenem resistant PA1004 Evo10. The Evo10 strain was created by evolving PA1004 through a serial passage in the presence of increasing concentrations of meropenem. For this, 100 µl of an overnight culture of PA1004 was spread onto LB agar containing 0.5 µg/ml meropenem and incubated overnight at 37°C. The following day a single colony was inoculated into LB containing 0.5 µg/ml meropenem. Overnight growth and plating were repeated as previously onto LB agar containing 1 µg/ml meropenem, followed by a single colony being inoculated into LB containing 1 µg/ml meropenem. These steps

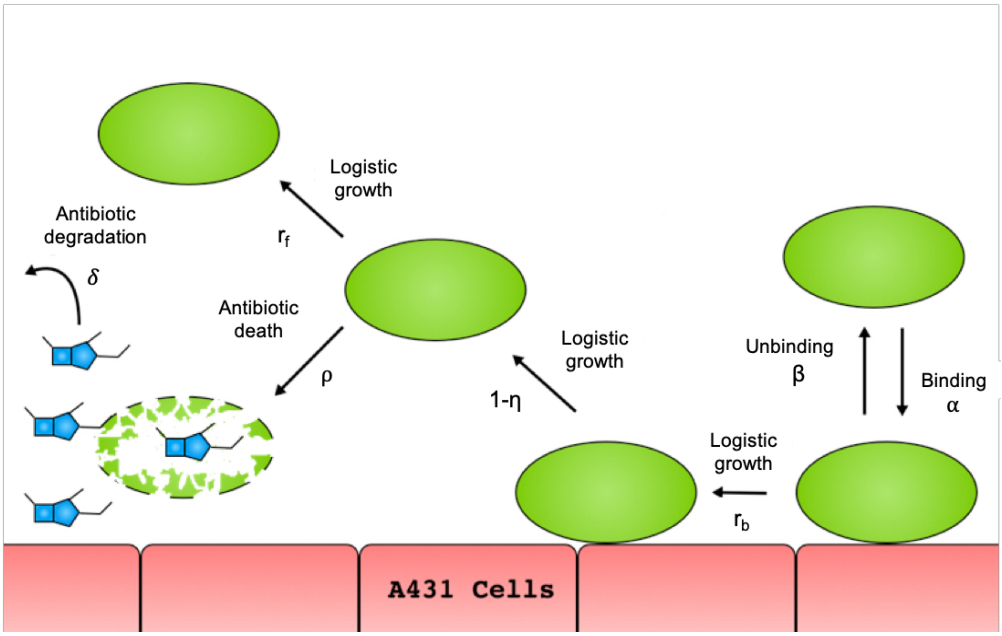


Fig 1. Diagram displaying structure of the model. Bacteria exist as free floating and bound to A431 cells and can move between these populations by binding (α) and unbinding (β). Both populations grow logistically (r_f and r_b) with a proportion of bound cells entering the free compartment ($1-\eta$), and both are killed by antibiotics (ρ) that degrade over time (δ).

were repeated up to 3 µg/ml and then in 1 µg/ml increments up to 10 µg/ml. At this point the strain was considered resistant as it was able to grow in concentrations above 8 µg/ml [100], and was named PA1004 Evo10.

2.3.2. Attachment

The attachment experiments were performed using human epidermoid carcinoma cell line A431. 2.5 x 10^5 cells were seeded to a 24 well plate, while bacterial cultures were grown overnight at 37°C with shaking at 200 rpm. The following day, the OD_{600} of each culture was measured and CFU calculated via a calibration curve. Wells were washed 3 times with PBS and an amount of culture diluted into Dulbecco's Modified Eagle's Medium (DMEM) such that the 0.5 ml inoculated into each well contained 2.5 x 10^6 bacterial cells. Another 0.5 ml of DMEM was added containing the required amount of meropenem. The plate was centrifuged at 1000 rpm for 5 minutes and incubated at 37°C.

To quantify the levels of free bacteria, the supernatant was removed at set time periods, diluted and plated onto LB agar. For the bound bacteria, wells were washed 3 times with PBS and then incubated with 1% Triton X-100 for 10 minutes at 37°C. The cell lysate was diluted and plated onto LB agar. Plates were then incubated overnight at 37°C before colonies were counted.

To parameterise the model, we used two sets of data, referred to as data sets 1 and 2 respectively. Each set contains data from experiments using PA1004 and Evo 10, both treated and untreated with meropenem. Time points used in data set 1 were 1, 2, 3, 4, 5, 6, 24 and 25 hours for PA1004, and 2, 4, 6, and 24 hours for Evo10. The treated experiments used 2 μg/ml meropenem for PA1004 and 75 μg/ml for Evo10. Data set 2 used time points 1, 2, 3, 4, 5, 6, 24, and 25 hours, with 5 μg/ml meropenem for both PA1004 and Evo10.

2.3.4. Growth curves for parameterisation

PA1004 was grown overnight in LB and diluted into both LB and DMEM to an OD_{600} of 0.4 the following day. 0.5 ml of culture and 0.5 ml of antibiotic were added to wells of a 24 well plate. OD_{600} readings were taken every 10 minutes, with the plate incubated at 37°C and shaken at 200rpm between readings. Antibiotic concentrations used were 0, 1, 2, 4, 8 and 16 μg/ml.

2.3.5. Calibration curve

PA1004 was grown overnight in LB and diluted to an OD_{600} of 1.0 the following day. Dilutions with an OD_{600} of 0.2, 0.4, 0.6 and 0.8 were then also prepared and serially diluted to be plated on LB agar. Colonies were counted, and CFU/ml calculated. The MATLAB *fminsearch* function (*fminsearch* is a local optimiser that explores parameter space in the neighbourhood of initial guesses) was used to generate a straight line with intercept of 0 using the least square method; giving 7.19×10^8 CFU = 1.0 OD.

2.3.6. Model formulation

An ODE model was created consisting of three elements: bound bacterial density, $B_b(t)$ (cells cm^{-2}); free bacterial density, $B_f(t)$ (cells cm^{-3}); and antibiotic concentration, $A(t)$ (antibiotic molecules cm^{-3}). The model is summarised in figure 1 and described by the following equations:

$$\frac{dB_b}{dt} = \underbrace{\big(1 + (\eta(E) - 1)H(K_b - B_b)\big) r_b B_b \left(1 - \frac{B_b}{K_b}\right)}_{\text{logistic growth (a proportion, } \eta, \text{ remain attached)}} + \underbrace{\alpha V B_f E}_{\substack{\text{binding} \\ \text{to human} \\ \text{cells}}} - \underbrace{\beta B_B}_{\substack{\text{unbinding} \\ \text{from human} \\ \text{cells}}} - \underbrace{\frac{\rho A}{A + A_{50}} B_b}_{\substack{\text{death due to} \\ \text{antibiotic}}},$$

$$\frac{dB_f}{dt} = \underbrace{r_f B_f \left(1 - \frac{B_f}{K_f}\right)}_{\text{logistic growth}} + \underbrace{\big(1 - \eta(E)\big) H(K_b - B_b) \frac{r_b}{h} B_b \left(1 - \frac{B_b}{K_b}\right)}_{\substack{\text{proportion of bound cells that become free} \\ \text{bacteria upon division}}} - \underbrace{H(K_b - B_b) \alpha A_r B_f E}_{\text{binding to human cells}}$$

$$+ \underbrace{\frac{\beta}{h} B_b}_{\substack{\text{unbinding} \\ \text{from human} \\ \text{cells}}} - \underbrace{\frac{\rho A}{A + A_{50}} B_f}_{\substack{\text{death due to} \\ \text{antibiotic}}},$$

$$\frac{dA}{dt} = - \underbrace{\delta A}_{\substack{\text{antibiotic} \\ \text{decay}}} - \underbrace{\frac{\sigma A}{A + A_{50}} B_b B_f}_{\substack{\text{irreversible binding} \\ \text{of meropenem to PBPs}}}.$$

It is assumed that the bacteria grow logistically, up to a carrying capacity of the system where growth becomes negligible. Logistic growth is a term that describes when the growth rate decreases as the population level gets closer to the maximum possible population, called the carrying capacity. We use mass action kinetics to model the rate at which the following processes occur: binding of free bacteria to A431 cells, unbinding of bound bacteria, antibiotic degradation and the irreversible binding of antibiotic to bacterial cells. We use growth rates r_b and r_f to model growth up to carrying capacities K_b and K_f for bound and free bacteria respectively. If the population of bound cells reaches

its carrying capacity then the growth term becomes a death term through use of a Heaviside step function $H(K_b - B_b(t))$ where

$$H(x) \coloneqq \begin{cases} 0 \; if \; x < 0 \\ 1 \; if \; x > 0 \end{cases}.$$

Table 1. Estimated and measured parameter values

Parameter	Description	Value	
		PA1004	**Evo10**
r_b	Intrinsic growth rate of bound bacteria (min^{-1})	$3.48x10^{-2}$	$3.48x10^{-2}$
r_f	Intrinsic growth rate of free bacteria (min^{-1})	$2.88x10^{-3}$	$2.88x10^{-3}$
K_b	Carrying capacity of bound bacteria (cells cm^{-2})	$7.27x10^{7}$	$7.27x10^{7}$
K_f	Carrying capacity of free bacteria (cells cm^{-3})	$2.71x10^{9}$	$2.71x10^{9}$
α	Binding rate of free bacteria to A431 cells (min^{-1} $site^{-1}$)	$8.79x10^{-11}$	$8.79x10^{-11}$
β	Unbinding rate of bacteria from A431 cells (min^{-1})	$1.53x10^{-10}$	$1.53x10^{-10}$
E_{init}	Density of binding sites (sites cm^{-2})	$9.76x10^{7}$	$9.76x10^{7}$
η_{max}	Maximum proportion of daughters of bound cells that can enter the bound compartment (dimensionless)	$1.79x10^{-3}$	$1.79x10^{-3}$
γ	Concentration of binding sites at which $\eta = \eta_{max}/2$ (sites cm^{-2})	$1.09x10^{4}$	$1.09x10^{4}$
δ	Intrinsic decay rate of antibiotic (min^{-1})	$5.13x10^{-3}$	$5.13x10^{-3}$
ρ	Death rate of bacteria due to antibiotic (min^{-1})	$4.29x10^{-2}$	$4.29x10^{-2}$
σ	Decay rate of antibiotic due to irreversible binding to bacteria (min^{-1})	$7.38x10^{-12}$	$7.38x10^{-12}$
A_{50}	Antibiotic concentration required for half maximal killing effect (μg ml^{-1})	$3x10^{-1}$	24.6
A_r	Area of the well (cm^2)	1.9	1.9
h	Height of culture media (cm)	$5.26x10^{-1}$	$5.26x10^{-1}$
V	Volume of culture media (cm^3)	1	1

Parameters α and β represent the rates of bacterial binding and unbinding respectively. The term $E(t)$ represents the number of binding sites available at time t such that $E(t) = E_{init} - B_b$, where E_{init} is the initial number of available binding sites. As bound cells reproduce, a proportion of their daughter cells become free bacteria due to either a lack of available binding sites, or the angle at which the cell divides. The proportion that remain bound is represented by $\eta(E(t))$. This is dependent on $E(t)$ such that more cells will remain bound the more empty binding sites there are available at time t. This is modelled using the following Hill function where η_{max} is the maximum proportion of daughter cells that can remain bound:

$$\eta(E) = \frac{\eta_{max} E^n}{\gamma^n + E^n}.$$

The antibiotic is assumed to degrade over time with rate δ and have a killing effect on bacteria with rate ρ. The concentration of antibiotic needed for half maximal killing is represented by A_{50}. Due to the mechanism of action of meropenem we assign σ to model the irreversible binding of antibiotic to the bacterial PBPs.

A scaling factor was initially employed to capture a potential difference in the antibiotic killing rate between bound and free bacteria, but parameterisation to experimental data identified this difference to be negligible and was subsequently ignored.

The model uses the dimensions of an individual well of a 24 well plate, such that A_r is the area of the bottom of a well, while h and V are the height and volume of 1 ml of liquid in each well respectively.

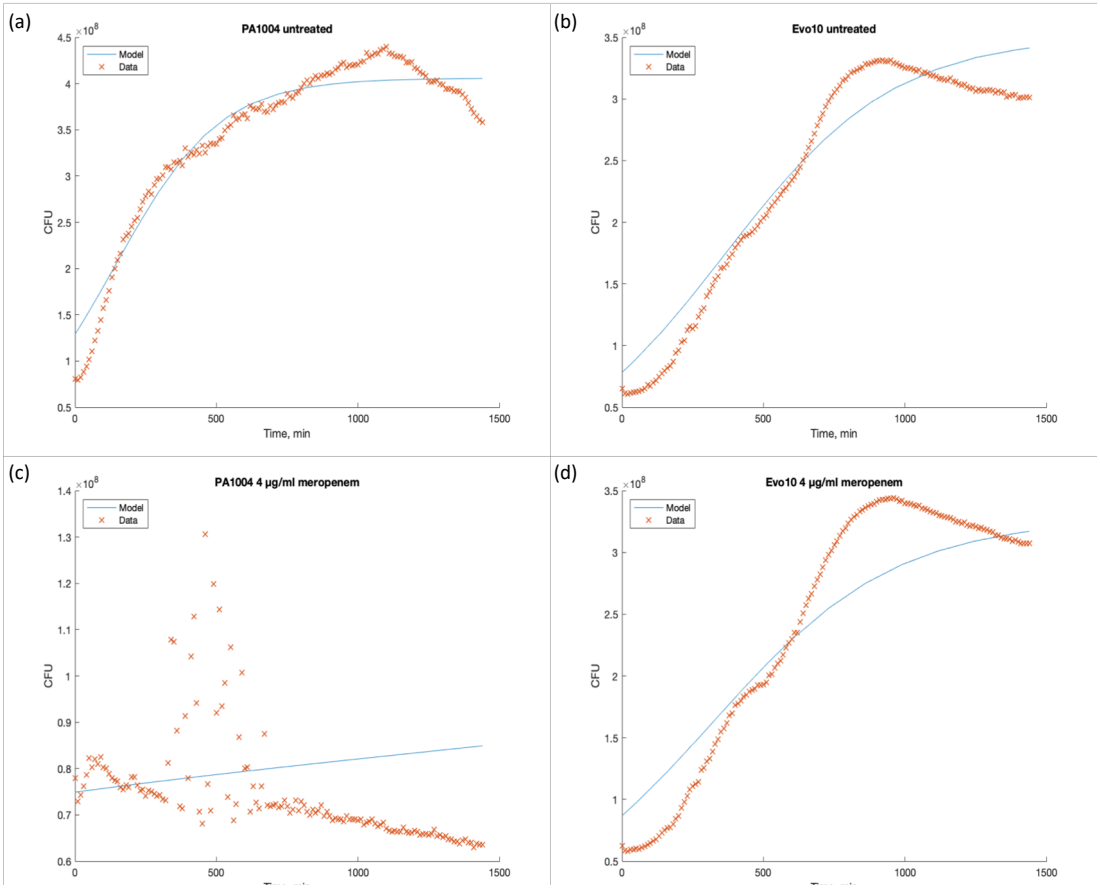


Fig 2. The solutions to the equation $\frac{dB_f}{dt} = r_f B_f \left(1 - \frac{B_f}{K_f}\right) - \frac{\rho A}{A+A_{50}} B_f$, plotted with experimental data for growth curves of a) PA1004, untreated, b) Evo10, untreated, c) PA1004, A=4 µg/ml, d) Evo10, A=4 µg/ml. From c), we show 4 µg/ml is able to drastically reduce population in PA1004, while d) Evo10 is unaffected. This result was consistent up to 16 µg/ml. The output in c) is unable to match the behaviour of the data as there is nothing in our model to generate the repeated rise and fall of the bacterial population seen around t=500. The model attempts to compromise between the growth in the middle and death at the end, so ends up with a poor fit overall. More complex growth and death terms may be required to accurately reflect this.

2.4. Results

2.4.1. Model parameterisation

Parameters were estimated by using the MATLAB function *fminsearch* to minimise the square of the error between the model output and experimental data. We were not able to use data from growth curve experiments performed

in DMEM for parameterisation as the growth did not display logistic behaviour and hence our model was not suitable (data not shown). The growth curves of PA1004 and Evo10 in LB without meropenem were used to estimate the parameters for intrinsic growth rate and carrying capacity of the free bacteria, r_f and K_f, as shown in figure 2(a),(b).

These values produced a good fit between the data and the model and were then used with the kill curves for all concentrations of meropenem to estimate parameters associated with the antibiotic, $\delta, \rho, \sigma \text{ and } A_{50}$.

All parameters estimated from the growth and kill curves were then fixed and used to generate values for parameters associated with growth of the bound bacteria, by comparison with experimental attachment data. However, these parameters were not able to replicate the results seen in the data. Instead, further parameterisation was performed with all parameters for PA1004 being estimated at once, using the estimated values from the growth and kill curves as initial guesses in the *fminsearch* function. This appears to show that the parameters are affected by the different experimental conditions, i.e. the change from growth in LB to growth in DMEM.

Using this method, we found that the parameter for the death rate of bacteria due to the antibiotic was too low to account for the drop in both bound and free populations seen in the experiments. Using a higher value led to a significant drop in population between treated and untreated bacteria after 25 hours, which is not seen in the experimental data. Using a higher guess for the death rate and intrinsic decay rate of antibiotic produced a parameter set that better fit the data.

These parameter values found for PA1004 were then used to estimate parameters for Evo10. As Evo10 is resistant to 5 μg/ml meropenem, we would have been unable to estimate parameters such as the death rate of bacteria due to the antibiotic. For this reason, the 75 μg/ml data was used alongside the untreated data instead. Our final parameter estimates are given in Table 1.

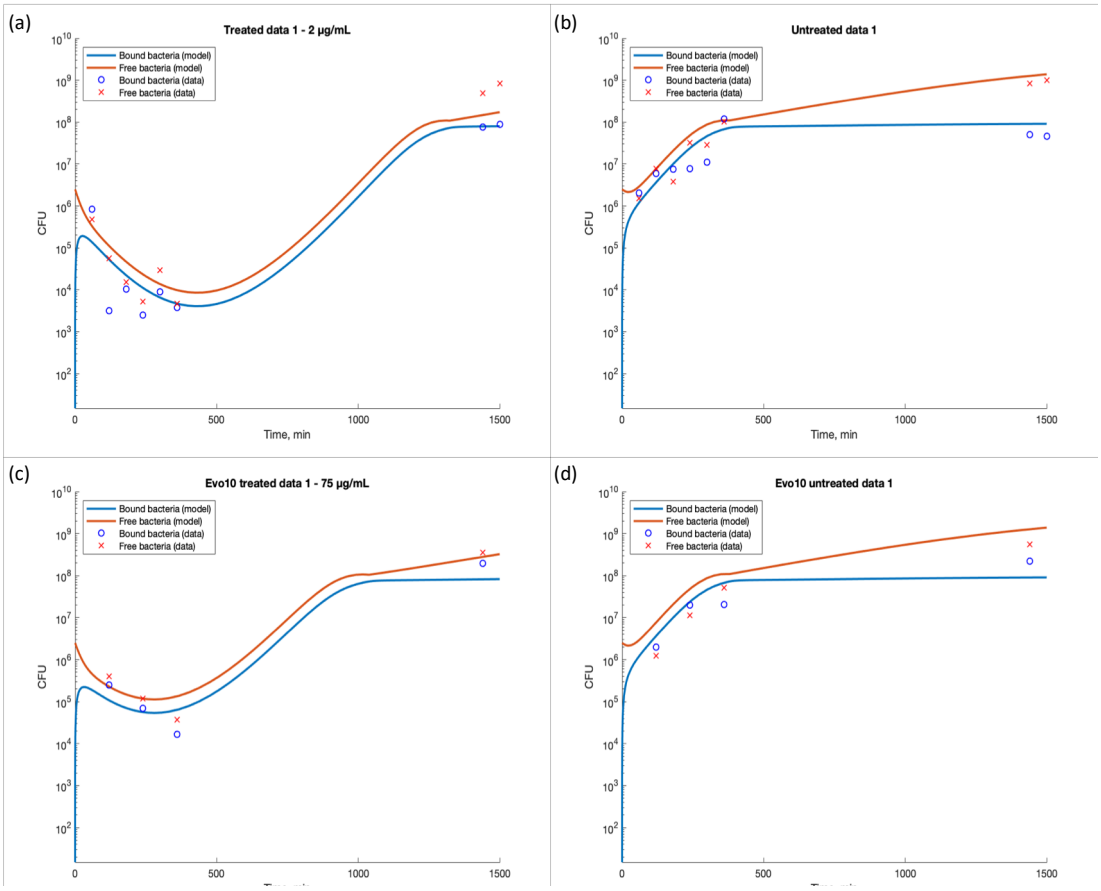


Fig 3. The solutions to the model with parameters from table 1, for a) PA1004 and 2 µg/ml meropenem, b) PA1004 untreated, c) Evo10 75 µg/ml, Evo10 untreated. These are plotted alongside data set 1 from the A431 attachment experiments. The units used are CFU per cm^2 for bound bacteria, and CFU per cm^3 for free bacteria.

The table shows the only difference between the parameter sets for experiments with PA1004 and Evo10 is the increased concentration of antibiotic required for half maximal killing (A_{50}), which is higher for the resistant strain as expected. That the parameters for intrinsic growth rates of both bound and free bacterial populations (r_b and r_f) are the same is interesting. It suggests that the fitness cost of resistance in Evo10 is negligible over this time period. We also note that the intrinsic growth rate of bound bacteria is an order of magnitude higher than that of the free bacteria. The proportion of cells that remain bound (η_{max}) is also very low and these

parameters combined mean the majority of the daughters of bound cells will become free bacteria.

2.4.2. Attachment results

Figure 3 shows the results of the parameterised model using the attachment data, data set 1. In the treated PA1004 experiments, both free and bound bacteria are significantly reduced in population size at $t < 500$ compared to the untreated experiments. By $t = 1500$ the populations have recovered to levels similar to those of the untreated bacteria. In the Evo10 experiments, we show that a strain evolved to be resistant to just 10 μg/ml meropenem shows very little difference in growth dynamics in the presence of 75 μg/ml meropenem.

We note that at some time points the data shows the bound bacteria at higher levels than the free bacteria and the model is unable to replicate this behaviour. However, for the majority of the data the free levels are higher than bound levels at each time point. Furthermore, the times at which the data shows numbers of bound bacteria higher than free bacteria are not consistent across the experiments. Overall, the model captures the overall trends of the data.

2.4.3. Parameter testing

To have confidence in our parameter values, we compared the model output to the results from data set 2, as these were not used in parameterisation. Figure 4 shows that although the model is not able to replicate the exact dynamics of growth, it does fit the overall behaviour of this data with a similar fit to that of data set 1. That the model is able to fit to data from separate experiments with differing antibiotic concentrations gives us high confidence of its usefulness.

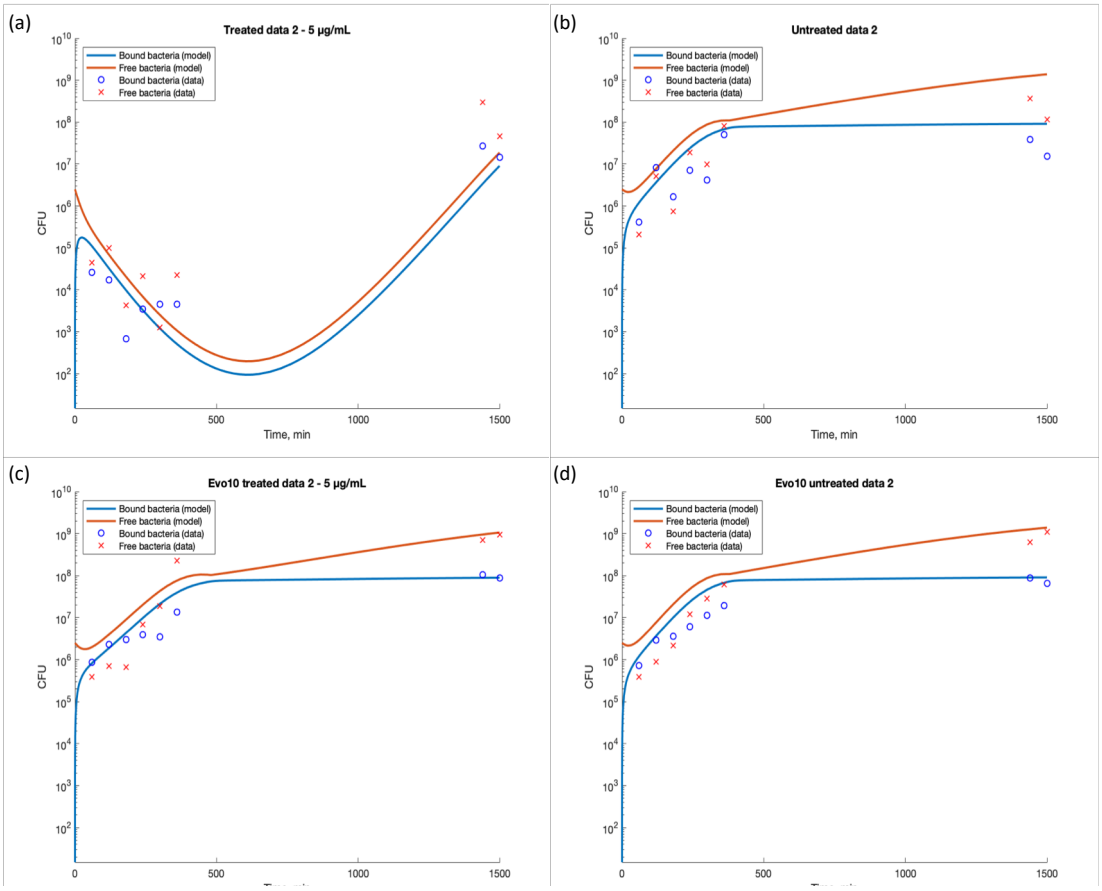


Fig 4. The solutions to the model with parameters from table 1, for (a) PA1004 and 5 µg/ml meropenem, (b) PA1004 untreated, (c) Evo10 5 µg/ml, (d) Evo10 untreated. These are plotted alongside data set 2 from the A431 attachment experiments. The units used are CFU per cm^2 for bound bacteria, and CFU per cm^3 for free bacteria.

2.5. Discussion

We present a model based on new experimental data that describes the growth of bound and free *P. aeruginosa* populations in the presence and absence of meropenem. Parameters used in the growth curves shown in figure 2 suggested a difference in intrinsic growth rate between the susceptible PA1004 and resistant Evo10 populations, with the PA1004 growth rate being slightly higher. However, further parameterisation in the attachment model showed no difference in the growth rates of these bacteria. We might expect the resistant strain to have a reduced growth rate due to the increased fitness

cost of resistance [101]. It is possible that the mutation(s) conferring resistance in Evo10 are no-cost mutations [102], or that the difference in growth rate was too small to be detectable over this time period [89].

The PA1004 growth curve experiments containing 4 μg/ml meropenem, as well as those containing higher concentrations, resulted in a greatly reduced bacterial population after 24 hours as seen in figure 2. This reduction in population is not seen in the attachment experiments containing 5 μg/ml meropenem. This shows the ability of *P. aeruginosa* to bind to the A431 cells is allowing the bacterial population to survive a concentration of antibiotic it would otherwise be at least inhibited by. It also highlights the limitations of basic *in vitro* experiments, as the more complicated attachment experiments produce different results.

Our model suggests that it is the *P. aeruginosa* bound to the A431 cells that drives the population recovery seen after 25 hours in the attachment experiments, due to their much higher growth rate. Consistent with Roberts *et al.* [98], we find the intrinsic growth rate of the bound bacteria is an order of magnitude higher than the same rate of free bacteria. Similarly, the parameter η_{max} is comparatively low, meaning the vast majority of the bound daughter cells do not remain on the A431 cell surface but are released to become free bacteria.

A recent study by Laventie *et al.* suggests that *P. aeruginosa* divides asymmetrically upon encountering a surface [103]. They explain that the daughter of a free-swimming cell that encounters the host surface becomes the first of our "bound" bacteria, while the parent cell detaches from the surface and becomes free once again. This asymmetric division then continues for several generations. While this has little implication for the model, as knowing the precise cell that becomes bound or free is not necessary when modelling the entire population, it does help explain the large proportion of daughter cells leaving the bound compartment. It appears it is not by chance, based on the axis of division of the cell, but an intentional strategy by *P. aeruginosa* to optimise virulence and tissue colonisation.

This experimental data and our model parameters predict that the ability of *P. aeruginosa* to bind to host cells enables the bacteria to survive higher concentrations of antibiotic than would otherwise be possible for a planktonic population. This behaviour in general could be attributed to the ability of *P. aeruginosa* to form biofilms. However, resistance to antibiotics due to biofilm formation is generally attributed to structural features and variant phenotypes within the biofilm [104–106], rather than the increased growth rates shown in this model.

The growth curves of PA1004 and Evo10 that were used to parameterise the model, as well as the OD to CFU calibration curve, were calculated using data from experiments with cultures grown in LB. We were not able to use data from the experiments in DMEM as it does not show logistic growth as is assumed in the model. Future work could include further repeats of these experiments to clarify this behaviour, as well as OD calibration experiments in DMEM, to better fit the model to the experimental conditions.

Future attachment experiments would ideally have more time points between the 6- and 24-hour mark than those in this project; as well as having more varied antibiotic concentrations. This would enable better parameterisation of the death rate of bacteria and degradation rate of the antibiotic, which were much higher than seen in the growth curve experiments.

As the process of adhesion is one of the most important factors in *P. aeruginosa* infection [83], understanding how our antimicrobial treatments interact with bound bacterial populations is an important step in tackling the problem of antimicrobial resistance. To our knowledge, this is the first experiment to model *P. aeruginosa* adhesion to human epidermal cells *in vitro*. We have successfully obtained a parameter set that is able to describe the growth of both antibiotic resistant and susceptible *P. aeruginosa* populations from multiple experiments with various antibiotic concentrations. This work paves the way for more complex studies of actual *in vivo* infections.

3. Looking at the world through the end of a municipal sewage pipe

3.1. Abstract

The rapid spread of transmissible extended-spectrum beta-lactamase (ESBL) producing *Enterobacteriaceae* has become a worldwide problem. Levels are increasing globally both in the community and in hospital settings. The aim of this study was to examine the bacterial populations that enter a local waste water treatment plant (WWTP), and to characterise the mechanisms of antimicrobial resistance (AMR) therein. Selective isolation of *Enterobacteriaceae* and PCR detection of *bla* genes show all (n=30) isolates harbour the CTX-M beta-lactamase, with many others additionally harbouring the TEM enzyme. Selected gene sequencing shows that bacteria we isolated produce beta-lactamases closely related to the CTX-M-15 and CTX-M-14 enzymes that dominate the current AMR landscape. Following on from this, we also isolated CTX-producing MDR *K. pneumoniae,* as well as further potential nosocomial pathogens, including *Stenotrophomonas* and *Aeromonas* species. This study shows that surveillance of AMR in bacteria found in WWTPs could be an important tool for detecting trends in AMR across human populations.

3.2. Introduction

Since the discovery of a transferable extended-spectrum beta-lactamase (ESBL) in Germany in 1983, *Enterobacteriaceae* producing these enzymes have become a major global threat in both healthcare and community settings [107,108]. Originally, in the 1980s and 90s, the enzymes SHV and TEM were the dominant beta-lactamases throughout the world, carried mainly by clones of *Klebsiella* [109]. Since the turn of the century, the CTX-M family of enzymes has spread quickly to become the dominant ESBL, carried mainly by a few dominant clones of *E. coli* [110–112].

The rapid spread of the CTX-M enzymes, now known as the CTX-M pandemic, is thought to be due to a number of reasons. Firstly, the success of particular *E. coli* clones, such as ST131 containing the CTX-M-15 enzyme, have greatly contributed [113]. At the same time, these enzymes are often associated with IS*Ecp1*-like mobile genetic elements, contained on plasmids, which have increased the spread through horizontal gene transfer [114–116]. Finally, the organisms producing these enzymes show high levels of co-resistance to other antibiotics, particularly to aminoglycosides and fluoroquinolones [117–119].

CTX-M type ESBLs were first reported in the UK in 2003, where a large proportion were found in the community, mainly causing community acquired urinary tract infections (UTIs) [120–122]. These often lead to complications, with around 75% of *E. coli* bacteraemias developing from community infections [123]. Therefore, knowledge of the spread of resistant bacteria into the community, and the genes associated with their transfer, is especially important.

For reasons such as this, one of the key recommendations of the Review on Antimicrobial Resistance (AMR) chaired by Jim O'Neill was for increased surveillance of AMR [58]. One way to achieve this is the analysis of the influent of waste water treatment plants (WWTPs), a concept first proposed in the 1970s [124,125]. Recent studies have shown that the increasing levels of AMR in the human population is mirrored by increasing levels of resistance found in local WWTPs [126,127].

In one study, high levels of vancomycin resistant enterococci (VRE) were found in WWTPs in Sweden, despite the low levels of VRE previously found in community and hospital settings [128]. This was followed a number of years later by a marked increase in the number of VRE cases of the same resistance type that was previously found in the WWTPs [129].

Other recent studies have found that WWTPs contain high levels of ESBL producing *E. coli,* predominantly of the CTX-M type [130,131]. Furthermore, as acquired carbapenem resistance spreads around the globe, mobile carbapenemase genes have been detected in waste water in both the US and UK [132–134]

These studies suggest that surveillance of bacterial populations in WWTPs can serve as a proxy for surveillance of clinically relevant bacteria carried within the human population. This information could be used to recognise current and future trends in the spread of AMR, potentially leading to better control and prevention of antimicrobial infections.

The aim of our study was to investigate the presence of specific AMR genes in the raw sewage of a small WWTP that serves both a local village with a population of around 2200, and a small university campus with over 2500 students. This site is particularly interesting as it allows the comparison of a stable population, the village, with a mobile and international population of the university campus.

The investigation was to look for specific ESBL genes by selective isolation followed by PCR detection, as well as documenting the resistance patterns of bacteria carrying these genes by antibiotic sensitivity testing. We attempted to see if a difference in the levels of resistance could be found between what appear to be separate communities of a rural and more international population respectively. We also attempted to find carbapenem resistant *Enterobacteriaceae* (CRE) in wastewater, through the use of selective media, with the intention of determining their mechanism of resistance and overall patterns of AMR.

3.3. Methods

3.3.1. Sampling and antibiotic sensitivity testing

Sampling was performed at the local WWTP, with samples taken from influent pipes coming from both a local village and university campus separately. Samples were taken in triplicate, at around 10am in the morning, and were kept at room temperature until testing was performed within 4 hours. Samples from separate pipes are referred to as C for campus and V for village respectively.

From each sample, 100 µl of waste water was spread undiluted onto CHROMagar™ ESBL agar plates and incubated overnight at 37°C. From each plate, 6 colonies that appeared pink on ESBL agar were streaked onto TBX agar (Oxoid) to further select for *E. coli*. Blue/green colonies on TBX agar were subcultured onto LB agar and subject to oxidase and indole spot testing, before being frozen via MICROBANK™ (Pro-Lab Diagnostics) at -80°C. Multiple isolates from campus samples did not appear as blue/green colonies, so the previous step was repeated until there were 18 isolates from both the village and campus respectively,

Antibiotic sensitivity testing was carried out using the disk diffusion method set out by the Clinical and Laboratory Standards Institute [135]. Antibiotic disks used were ampicillin (10 µg), amoxicillin-clavulanic acid (20 µg/10 µg), cefoxitin (30 µg), cefotaxime (30 µg), cefpodoxime (10 µg), cefquinome (30 µg), aztreonam (30 µg), imipenem (10 µg), streptomycin (10 µg), oxytetracycline (30 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg), sulfamethoxazole-trimethoprim (23.75 µg/1.25 µg), chloramphenicol (30 µg), nitrofurantoin (300 µg) and azithromycin (15 µg), all purchased from Pro-Lab Diagnostics.

3.3.2. PCR detection of *bla* genes

DNA extraction was carried out using a boiled-cell method, where 2 or 3 colonies were inoculated into 100 µl TE buffer and heated for 30 minutes at 100°C. This was followed by centrifugation at 13000 g for 15 minutes and the resulting supernatant was transferred to a fresh microcentrifuge tube and stored at -20°C.

PCR was performed using a Bio-Rad C1000 thermal cycler, using DreamTaq Green PCR Master Mix (2X) (Thermo Fisher Scientific), with primers shown in table 2. Conditions for $bla_{CTX\text{-}M}$ were 94°C for 5 min, 30 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 1 min, and finally 72°C for 7 min. Conditions for bla_{TEM} were 95°C for 3 min, 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 m, and

Table 2. Primers used in PCR reactions.

Primer name	Sequence
blaCTX-M F	CGC TTT GCG ATG TGC AG
blaCTX-M R	ACC GCG ATA TCG TTG GT
blaTEM F	GAG TAT TCA ACA TTT CCG T
blaTEM R	TAA TCA GTG AGG CAC CTA T
16S rRNA 8F	AGA GTT TGA TCC TGG CTC AG
16S rRNA 805R	GAC TAC CAG GGT ATC TAA TC

finally 72°C for 5 m. The same conditions were used for *bla*$_{SHV}$ except that an annealing temperature of 54°C was used. Results were visualised using gel electrophoresis [136].

3.3.3. Gene sequencing

Of the isolates positive for *bla*$_{CTX-M}$, 15 were selected to have this gene sequenced by Eurofins genomics TubeSeq service. PCR was again used, this time with Q5® high-fidelity DNA polymerase (New England Biolabs), with conditions of 98°C for 30 s, 35 cycles of 98°C for 10 s, 65°C for 15 s, 72°C for 20 s, and finally 72°C for 2 min. Using isolate V1 alone, DNA was then purified using a Monarch® PC & DNA cleanup kit (New England Biolabs) according to the manufacturer's instructions. The concentration of DNA was determined using a NanoDrop® ND-1000 Spectrophotometer, followed by dilution with water according to the Eurofins TubeSeq instructions, to be collected for automated Sanger sequencing of both forward and reverse reads. After receiving the sequences of the V1 *bla*$_{CTX-M}$ gene, a further 14 isolates were chosen to be sent away for sequencing. Isolates were chosen based on their antibiotic sensitivity results, such that those with different patterns of resistance would be tested.

PCR was again performed using Q5® DNA polymerase on those 14 isolates as described previously, however only 4 showed up positive. All 14 samples showed as positive when PCR conditions used were 94°C for 5 min, 30 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 1 min, and finally 72°C for 7 min. The PCR products were purified and sent for sequencing, with both forward and reverse reads, as described previously.

For each isolate, the reverse complement of the reverse strand was determined using an online tool [137], followed by multiple sequence alignment using ClustalW [138]. Consensus sequences were determined where both reads overlapped, and subject to NCBI's BLAST to determine CTX-M-type. Sequences were then aligned using ClustalW multiple alignment in MEGA7.

3.3.4. Sampling and detection of carbapenem resistant bacteria

Water samples were taken as described previously. 50 ml of each sample was passed through a 45 µm filter and this was placed onto Brilliance CRE agar (Oxoid) to be incubated overnight at 37°C. Additionally, 100 µl was spread onto separate CRE agar plates and also incubated overnight. The plates were overgrown with bacteria and so various colonies were picked off to be incubated on further CRE agar overnight. Single colonies were then transferred to LB agar and incubated overnight. In total, 36 pure cultures were isolated, and AST, DNA extraction and freezing for long term storage was performed on these as described previously.

3.3.5. 16S sequencing and detection of *bla*$_{CTX\text{-}M}$ genes

Isolates were identified by sequencing the 16S ribosomal RNA gene and using NCBI's BLAST to determine the species. PCR was performed using Q5® DNA polymerase with the 8F and 805R 16S primer pair [139]. Conditions used were 98°C for 30 s, 35 cycles of 98°C for 10 s, 59°C for 15 s, 72°C for 20 s, and finally 72°C for 2 min. PCR products were purified and sent away for Eurofins TubeSeq

service as described previously, though only forward reads were used on this occasion.

PCR was also performed on all 36 isolates to detect the presence of *bla*$_{CTX-M}$ using DreamTaq DNA polymerase and conditions as described previously.

3.4 Results

3.4.1. Sample identification and antibiotic sensitivity testing

29 of the 36 isolates from ESBL agar were identified presumptively as *E. coli*, based on indole and oxidase results after growth on TBX agar; 1 was presumptively identified as *Klebsiella* sp., 5 were unidentified and 1 was discarded due to contamination. All isolates were resistant to ampicillin and cefpodoxime; all *E. coli* and *Klebsiella* isolates were resistant to cefotaxime, while high levels of resistant or intermediate results were seen to amoxicillin-clavulanic Acid (28/30) and cefquinome (29/30). Resistance patterns of the village and campus isolates can be seen in figure 5.

3.4.2. PCR detection of *bla* genes

The PCR results for the ESBL genes showed that 30 of 35 isolates were positive for *bla*$_{CTX-M}$, 12 of 35 positive for *bla*$_{TEM}$, and none positive for *bla*$_{SHV}$; all isolates with *bla* genes were presumptive *Enterobacteriaceae*. Of the 12 positives for *bla*$_{TEM}$, 10 were from the village influent while only 2 were from the campus.

3.4.3. Gene sequencing

Of the 15 DNA samples sent for sequencing, only 5 passed the Eurofins QC system, 6 failed both forward and back reads, and 4 came back with “short” results of less than 100 bp. However, by forming a consensus sequence where both forward and reverse reads showed the same result, which was possible even on sequences that failed QC, we were able to get sequences for 12 of 15 isolates. The BLAST results of these sequences showed that 4 (2 from the village

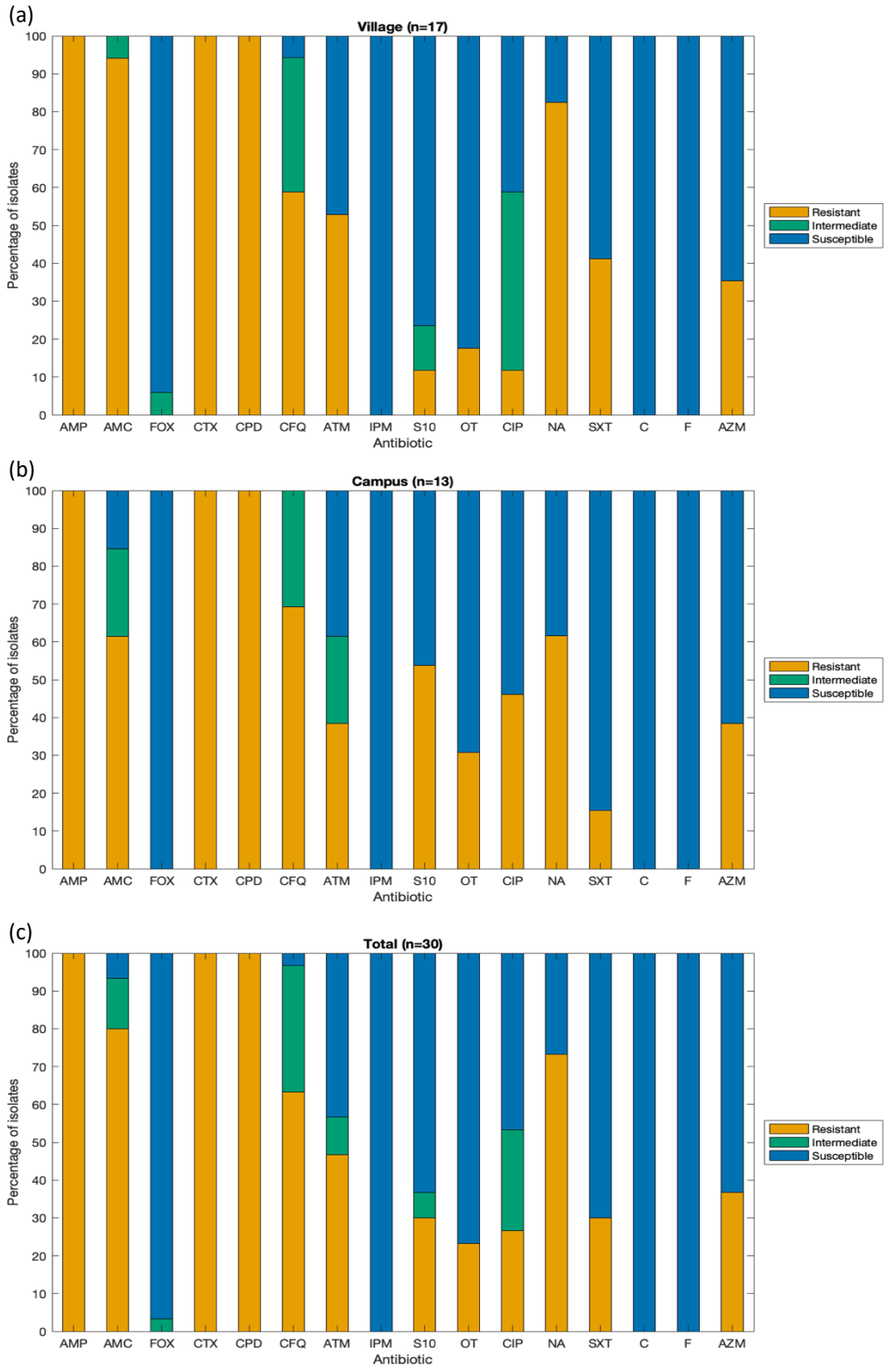


Fig 5. Levels of resistance in a) village, b) campus and c) total presumptive *Enterobacteriaceae* samples, shown as percentages. High levels of beta-lactam resistant can be seen across both populations, with overall levels of resistance similar between the village and campus.

and campus respectively) are most similar to CTX-M-15 and show exact homology with the NCBI CTX-M-15 reference, NG_048935.1, over a sequence of 441 bp [140]. The remaining 8 (4 from village and campus respectively) are most similar to CTX-M-14 and have 4 or less SNPs over a shared sequence of 393 bp, with 3 isolates showing complete homology to NCBI reference NG_048929.1 [141].

3.4.3. Detection of carbapenem resistant bacteria

Of the 36 isolates cultured from Brilliance CRE plates, 33 had their 16S gene successfully sequenced. BLAST searches revealed that 11 were most similar to *Stenotrophomonas maltophilia*. However, since *Stenotrophomonas* spp. cannot be reliably distinguished by 16S sequencing [142], these will be referred to as *Stenotrophomonas*.

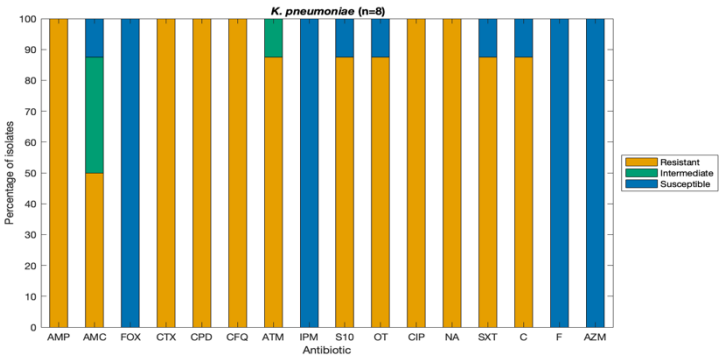
Of the remaining 25, 11 were identified as *Aeromonas* (not identified to species level), 8 as *K pneumoniae* and 3 as *Acinetobacter junii.*

The results of antibiotic sensitivity testing can be seen in figure 6. Not all antibiotics used are recommended tests for *Stenotrophomonas* spp., *Aeromonas* spp. or *Acinetobacter* spp., therefore only recommended tests are listed. The zone diameters of all antibiotics used in this study can be found in the supplementary information.

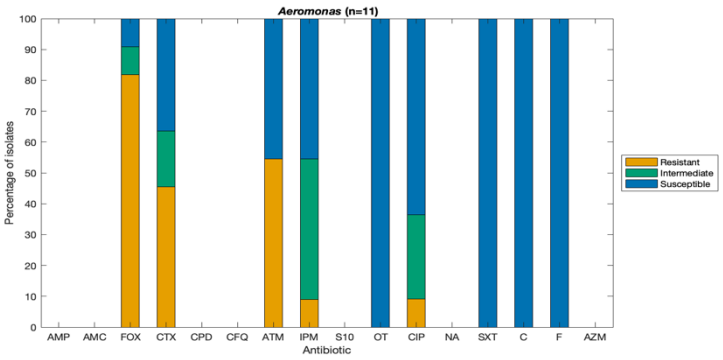
Carbapenem resistance was detected in 7 *Stenotrophomonas* and 1 *Aeromonas* isolate, while 4 other *Aeromonas* isolates gave intermediate results. An attempt was made to test for carbapenemase production via the Modified Hodge test [143], though this failed due to lack of a positive control.

The *K. pneumoniae* isolates showed the highest levels of AMR, with half the isolates being resistant to 12/16 antibiotics tested. *Aeromonas* isolates showed high levels of resistance to beta-lactams, with 10/11 having intermediate or resistant results to at least one cephalosporin tested. The only antibiotic tested that is recommended for use by the CLSI to treat *Stenotrophomonas* infections

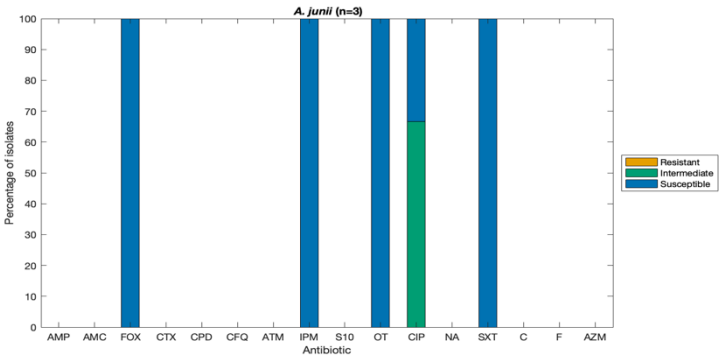
(a)



(b)



(c)



(d)

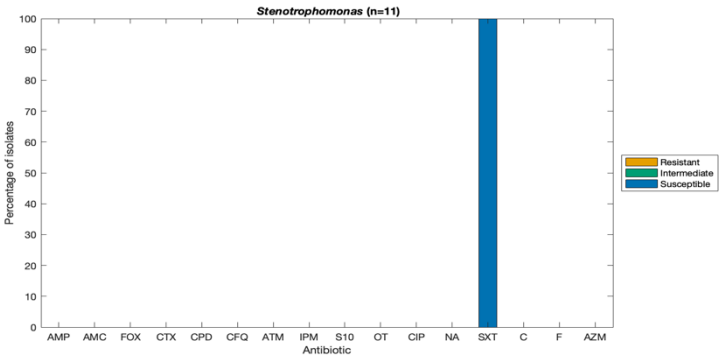


Fig 6. Levels of resistance of bacteria isolated using Brilliance CRE agar. Levels shown as percentages. Bar left blank for consistency when no zone diameter data for resistance available from CLSI.

High levels of resistance seen across a) *K. pneumoniae* isolates, with resistance or intermediate result to 12 of 16 antibiotics in majority of isolates. High levels of resistant or intermediate results found in b) *Aeromonas* species, while c) *A. junii* and d) *Stenotrophomonas* show no resistance, where antibiotics tested have zone diameter information available.

is sulfamethoxazole-trimethoprim, and all 11 isolates were susceptible. Similarly, the *A. junii* isolates did not show resistance to any of the recommended antibiotics tested.

PCR detection of $bla_{CTX\text{-}M}$ showed that this gene was present in 7/8 *K. pneumoniae* isolates.

3.5. Discussion

The AST results show little difference in antibiotic sensitivity between the *E. coli* isolates from the village and campus respectively. Similarly, all species isolated on Brilliance CRE agar were found in both village and campus samples. The only difference of note was the majority of village isolates harbouring both CTX-M and TEM beta-lactamases (58%, n=10/17), compared to the campus isolates (15%, n=2/13). We note that further sampling is required to determine if the bacterial populations are truly different. Though this study may suggest they are mostly similar, the low sample size prevents major conclusions from being drawn.

Globally, the most common $bla_{CTX\text{-}M}$ genes are $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}14}$, with $bla_{CTX\text{-}M\text{-}27}$ increasingly on the rise in many countries [144]. Recent studies in the UK have found similar results. A study comparing waste water samples and bloodstream infections found these three genes as being the most common in both environments [123]. Another study found $bla_{CTX\text{-}M\text{-}15}$ as the most common ESBL in *E. coli* infections in the UK, accounting for 95% of all infections, with $bla_{CTX\text{-}M\text{-}9}$-like genes (a group that includes both $bla_{CTX\text{-}M\text{-}14}$ and $bla_{CTX\text{-}M\text{-}27}$) making up the remainder[121]. In our study we find the 66.6% of those $bla_{CTX\text{-}M}$ genes sequenced to be most similar to $bla_{CTX\text{-}M\text{-}14}$, with the remaining 33.3% similar to $bla_{CTX\text{-}M\text{-}15}$. We note that the short lengths of our sequences, being 400-524 bps, make exact identification impossible when compared to the 824 bps of the NCBI reference for $bla_{CTX\text{-}M\text{-}15}$. Especially since the $bla_{CTX\text{-}M\text{-}14}$ and $bla_{CTX\text{-}M\text{-}27}$ genes differ in only a single nucleotide, at a position not sequenced in any of our isolates, meaning we are unable to determine their precise CTX-

M-type [144]. However, given everything we know of the spread of CTX-M-types around the globe, as well as their detection in environments such as those sampled in this project, this is evidence that our results align with those of previous studies.

As we were using media to select for *Enterobacteriaceae*, we did not intend to find many of the bacterial species that were grown on Brilliance CRE agar. *Stenotrophomonas* species are environmental bacteria found in aqueous environments, with intrinsic resistance to beta-lactamases including carbapenems via a chromosomal metallo-beta-lactamase [146]. Previously these bacteria have also been isolated from wastewater [147]. The type species *S. maltophilia* is an opportunistic nosocomial pathogen, mainly causing respiratory infections in patients with cystic fibrosis or those who are immunocompromised. However, all 11 isolates found in this study were susceptible to the first recommended antibiotic by the CLSI, sulfamethoxazole-trimethoprim. This may suggest that these isolates are not clinically relevant in terms of acquired multidrug resistance, however they still potentially pose a threat with their ability to cause intrinsically drug resistant infections.

Similarly, previous studies have found *Aeromonas* species to be ubiquitous in aqueous environments, as well as being found in the human gut, with high levels of resistant strains found in WWTPs [148–150]. It is an emerging nosocomial pathogen, being responsible for gastroenteritis as well as skin infections and bacteraemia. As well as beta-lactamase production, other studies have found high levels of tetracycline and sulfamethoxazole-trimethoprim resistance in drug-resistant strains of *Aeromonas* [149–151]. In this study, we found high levels of beta-lactam resistance, as well as over half of the isolates (6/11) having either an intermediate or resistant result to imipenem. Further work is needed to determine whether the mechanism of resistance of *Aeromonas* isolates is due to acquired beta-lactamase production, or some other factor.

A. junii is a rare human pathogen, associated with hospital acquired septicaemia [152–154]. *Acinetobacter* species can be human commensals, while they are also found in water sources in the environment, and many species of

Acinetobacter have been isolated from wastewater treatment plants in the past [155]. However, the lack of resistance to any of the front-line antibiotics that were tested implies these isolates may not be clinically relevant sources of AMR.

Further characterisation of the *Enterobacteriaceae* isolates could be carried out, including the determination of the sequence type (ST) of our *E. coli* isolates. Since it appears that the carriage of the $bla_{CTX\text{-}M\text{-}15}$ gene in the particularly successful *E. coli* ST131 has helped it spread to become the dominant ESBL found across the world, determination of ST would be a useful part of AMR surveillance [113].

Especially important is the determination of the role of MDR mobile genetic elements in the level of AMR seen in our results, especially in the *K. pneumoniae* isolates, where resistance is seen to almost all antibiotics tested. The presence of $bla_{CTX\text{-}M}$ genes in these isolates indicates the likelihood of an MDR plasmid being present [156]. Their presence could be confirmed through conjugation experiments, or whole-genome sequencing (WGS) techniques, to begin to characterise the mechanisms of the transfer of AMR genes in these bacteria.

In conclusion, this study provides evidence that surveillance of bacterial populations from WWTPs can be used to screen for antimicrobial resistant bacteria that will be present in the human population. This will help to provide clinicians with up to date information regarding the spread of AMR in their surrounding areas. It also has the potential, especially with the ever-decreasing cost of whole-genome sequencing, to act as an early warning system for emerging resistance.

4. General Discussion

The projects presented in this report were designed to address specific themes within the field of AMR. The first project focused on unconventional approaches to eradicating bacterial pathogens, through the use of mathematical modelling of *in vitro* experiments, as well as bacterial evolution of AMR. It was able to show that the growth rates of both susceptible and resistant strains of *P. aeruginosa* in the experiments were identical, implying that the mechanism of resistance in Evo10 does not affect bacterial fitness. It is possible that the mutations conferring resistance would have a detrimental effect on the growth rate in other growth conditions, or over longer time periods. Further investigation into the mechanism of resistance in Evo10 would be beneficial. Given the method of producing the resistant strain it seems likely that resistance is due to adaption of existing mechanisms, possibly through loss of OMPs or over expression of efflux pumps [18,41]. WGS of the genomes of both strains could be used to determine the resistance mechanism, potentially coupled with analysis of gene or protein expression [157].

Further mathematical analysis of the model should be carried out to assess its fitness, such as analysis of the steady states of the system, as the *fminsearch* function is only a local optimiser. This means it determines whether a new parameter value is a better or worse fit than a previously tested local value, starting with the original estimated values. As a way of explaining this, consider the equation $x^2 = 4$, it has two solutions in x = 2 and x = -2. A local search of the solution to an initial guess of x = 1 would determine the answer is x = 2. Since x = 0 is a worse solution than x = 1, a local function would not continue to search in that direction, so the solution x = -2 would not be found. It is therefore possible that another parameter set exists that also fits the experimental data. Consider a simple example of a system with only growth and death rates. A low growth rate and low death rate would give similar results to a high growth rate and high death rate, but these parameter sets would give vastly different

outcomes when other factors are then included. For these reasons, further work is required to test the accuracy of the model.

The results obtained are specific to the particular strains used, PA1004 and Evo10. One avenue to explore would be further experiments using different strains of *P. aeruginosa* to see if the model is able to be applied more generally. Since clinical isolates of *P. aeruginosa* display either cytotoxic or invasive phenotypes [158], There could be the possibility of exploring the difference this phenotype would have on the model, as an invasive phenotype would potentially have very different interactions between "bound" and free populations, as well as antibiotics, than a cytotoxic one.

Performing the original experiments using differing initial levels of bacteria would give a further data set that could be used to test the predictions of the model. Additionally, determining the levels of bound and free bacteria at time points between 6 and 24 hours in further experiments could generate further data to test the model.

The system could also be expanded to include other additional mechanisms of bacterial resistance to antibiotics such as persister cell formation, further adapted from the modelling work done by Spalding *et al.* [99,159].

Overall the project achieved its aims and was able to explain behaviours of the bacterial population that might not be obvious, such as the growth rate of bound bacteria being the driving force of the population growth. This implies that prevention of *P. aeruginosa* binding to host cells could be a potential method of reducing the levels of infection. The idea of producing therapeutics to target virulence mechanisms rather than bactericidal ones is a relatively new and unconventional one [160,161], that has the potential benefit of delaying the emergence of resistance to new drugs. This is considered a possibility because the compounds would not kill the bacteria, only disarm them, enabling the host immune response to clear the infection. The selection pressure exerted by these drugs would in theory be lower than that of conventional antibiotics, and so resistance should take longer to form. Another advantage of the strategy of disarming virulence is that it creates a vast array of targets for new drugs.

Though this necessarily creates narrow-spectrum therapeutics, if combined with advances in diagnostic technology it could lessen the need for broad-spectrum antibiotics, which would in turn slow the evolution of resistance to our drugs of last resort.

The second project described in this report focused on the themes of genomic epidemiology and bacterial evolution of AMR within the context of a WWTP. It aimed to discover the types of ESBL-producing *Enterobacteriaceae* that could be isolated from the separate influent pipes. Other goals were to determine the role of plasmids in the resistance mechanisms of these bacteria, as well as find out if there were differences in the resistance profiles between bacterial populations from the local village and university campus. Using the influent rather than effluent means the populations sampled should be representative of the bacteria within the local human populations, and not those that proliferate within the WWTP itself.

The project showed that the levels of resistance of ESBL-producing *Enterobacteriaceae* in both the village and campus were broadly similar. Determination of their $bla_{CTX\text{-}M}$ types found that both populations contained the same types of $bla_{CTX\text{-}M}$ genes and at similar levels. It revealed the prevalence of $bla_{CTX\text{-}M}$ genes overall, as all but one of the 38 *Enterobacteriaceae* found in the study contained this gene. This could be due to the sampling method using specific ESBL and CRE selective agars. Another approach could be to use less selective agars and determination of ESBL-producing phenotype from ASTs, followed by PCR detection of ESBL genes.

Unfortunately, due to the time constraints of a 15-week project, the work of isolating plasmids carried by the ESBL-producing bacteria could not be accomplished. It was intended that the project would involve the horizontal transfer of resistance plasmids into recipient strains, and the resistance phenotypes provided by the plasmid would then be determined. This could be combined with techniques such as inverse PCR[162] to discover other mobile elements involved in the transfer of AMR genes, such as is the case with CTX genes and IS*Ecp1* element.

If this project were carried on at PhD level, it would allow for a very large data set to be established. The ease of access to the WWTP and the method of sampling potentially allows for the screening of a very large number of bacteria. This could also be combined with WGS of a subset of bacterial genomes to get a broader picture of the total AMR genes present. There is also potential to use techniques such as Hi-C-based metagenomics to determine the overall levels of AMR and their presence within the bacterial genomes, without having to use selection methods that may alter the results [163].

Another factor to consider is the potential virulence of the cultured strains. Antibiotic resistance alone does not imply that the isolated bacteria will be a direct clinical threat [164]. Further work could include determination of whether the bacteria harbouring these AMR genes are also capable of causing infection themselves, or whether they are simply carriers of AMR and an indirect threat via proliferation of AMR genes to virulent strains by HGT. PCR detection of virulence genes could be used, although as pathogenicity is often a complex process, it may be difficult to assess virulence in environmental samples by this method alone. Techniques such as reverse dot-blot hybridisation[165] or WGS could be used to better assess the potential for virulence.

Overall, the project was a useful starting point in the determination of AMR genes present within the WWTP. The surveillance of AMR is of critical importance as it enables the tracking of resistance mechanisms throughout human and animal populations. As shown with the cases of specific beta-lactamases, such as the CTX-M-15 and NDM-1 enzymes, particular AMR genes can spread quickly throughout populations to become dominant at a nationwide, or even higher, level. Surveillance initiatives enable clinicians to be better informed of the AMR genes present in local populations and offer the correct treatment accordingly. They also enable the early detection of resistant strains that could pose a clinical threat in future, and allow us to determine the effects of changes in policy to combat AMR [166].

4.1. Concluding remarks

Although it could be said that AMR is simply caused by bacterial selection pressures to antibiotics, in many ways it is our misuse and misunderstanding of those selection pressures that has caused the problem we now face. As outlined by the numerous reviews and action plans over the last few years, the AMR crisis can only be solved by dealing with its myriad of causes at once. Solutions must include dealing with the improper use of antimicrobials, both in humans and animals; surveillance of AMR genes and directed use of that surveillance; prevention of microbial infections; as well as research into new diagnostics, therapeutics and vaccines[167]. This requires collaboration between various scientific disciplines, businesses, governments and international organisations. The two projects described in this thesis display the importance of a multifactorial approach to combating the threat of AMR. Although we can never fully solve the problem, as microorganisms will always continue to evolve, there is hope that working together we will manage to avert the disaster of a post-antibiotic world.

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6. Supplementary information

Table 3. Zone diameter sizes, mm, for village isolates. All presumptive *E. coli* except for V11, presumptive *Klebsiella.*

ID	AMP	AMC	FOX	CTX	CPD	CFQ	ATM	IPM	S10	OT	CIP	NA	SXT	C	F	AZM
V1	0	7	21	0	0	0	0	30	18	25	29	8	0	31	21	9
V2	0	11	24	8	0	16	13	32	18	22	35	26	29	30	20	25
V3	0	10	25	0	0	11	9	33	17	22	28	8	0	31	24	13
V4	0	11	25	9	0	17	13	31	16	23	30	26	29	28	24	19
V5	0	8	21	9	0	21	11	30	18	25	23	0	26	27	21	18
V6	0	11	28	10	0	20	17	36	17	25	25	9	27	32	22	13
V7	0	11	24	14	8	23	24	32	17	25	25	0	0	31	24	10
V9	0	11	28	15	0	20	21	34	19	32	26	0	26	30	23	15
V10	0	14	25	12	0	17	23	34	0	9	0	0	26	29	23	13
V11	0	9	17	14	0	19	25	32	12	0	23	0	0	28	23	12
V12	0	9	25	0	0	15	14	34	15	27	31	26	29	30	21	16
V13	0	11	20	0	0	18	21	28	0	8	0	0	25	28	21	21
V14	0	0	19	16	6	22	30	32	15	29	23	0	0	27	22	11
V15	0	0	20	16	0	21	28	31	15	28	23	0	0	27	21	11
V16	0	8	21	9	0	19	13	29	18	28	25	0	27	27	22	17
V17	0	11	21	16	0	20	27	30	14	28	22	0	0	28	21	11
V18	0	7	20	10	0	19	14	29	17	28	26	0	26	29	21	16

Table 4. Zone diameter sizes, mm, for campus isolates. All presumptive *E. coli* except for C45, C47-C51, unknown identification

ID	AMP	AMC	FOX	CTX	CPD	CFQ	ATM	IPM	S10	OT	CIP	NA	SXT	C	F	AZM
C1	0	14	27	11	0	17	18	30	9	9	0	0	25	28	23	11
C3	0	12	24	9	0	15	12	30	17	24	32	25	28	30	22	18
C4	0	10	24	16	7	21	25	34	15	23	26	0	0	29	23	10
C7	0	12	26	9	0	17	11	34	17	24	33	26	27	31	21	17
C9	0	12	25	10	0	16	12	32	17	24	35	26	27	31	20	26
C10	0	17	25	16	0	21	20	32	8	25	27	24	26	31	20	20
C11	0	12	27	10	0	18	18	32	10	8	0	0	24	30	19	10
C12	0	8	19	0	0	0	7	30	10	28	26	7	0	29	23	8
C17	0	8	18	10	0	18	15	23	15	26	21	0	21	29	20	16
C18	0	20	22	17	0	22	25	33	0	31	26	27	27	35	22	22
C38	0	15	22	10	0	17	22	32	0	9	0	0	25	32	21	12
C44	0	23	26	0	0	17	23	32	0	0	0	0	23	29	21	19
C45	0	0	0	23	0	27	31	28	0	29	20	0	18	30	24	18
C46	0	10	20	17	0	20	29	33	15	28	0	0	28	27	23	18
C47	0	0	0	23	0	28	35	28	9	33	25	0	20	33	27	19
C48	0	0	0	23	0	27	34	29	8	30	29	8	23	31	27	18
C49	0	0	0	24	0	29	37	31	0	33	25	10	22	33	26	19
C51	0	10	0	18	0	29	20	31	25	30	33	13	11	22	0	13

Table 5. Zone diameter sizes, mm, for village and campus isolates, organised by 16S sequencing result.

16S	ID	AMP	AMC	FOX	CTX	CPD	CFQ	ATM	IPM	S10	OT	CIP	NA	SXT	C	F	AZM
Aeromonas	**V19**	0	0	0	30	0	31	31	21	12	30	21	0	23	33	25	21
Aeromonas	**V20**	0	0	0	33	27	31	32	23	12	33	21	12	25	33	28	22
Aeromonas	**V22**	0	0	0	18	0	25	14	23	0	31	20	9	22	33	28	21
Aeromonas	**V23**	0	0	0	23	11	28	13	21	11	31	15	9	22	31	25	18
Aeromonas	**V24**	0	0	0	34	26	32	32	25	14	31	23	12	24	34	27	22
Aeromonas	**V27**	0	0	8	19	10	25	9	24	0	32	23	13	25	35	30	25
Aeromonas	**V30**	0	0	15	23	23	26	31	21	0	23	20	9	21	25	23	0
Aeromonas	**C53**	0	10	10	16	11	28	14	23	0	31	23	11	22	31	25	25
Aeromonas	**C54**	0	10	10	17	10	23	12	22	0	32	28	11	23	31	30	0
Aeromonas	**C55**	0	0	8	17	9	24	13	22	8	28	22	0	23	34	26	24
Aeromonas	**C60**	0	12	21	32	25	28	30	19	0	20	17	0	21	23	25	17
Klebsiella	**V21**	0	0	25	10	0	14	14	31	0	0	16	0	0	0	23	15
Klebsiella	**V26**	0	8	23	11	0	14	10	29	0	7	16	0	0	0	23	14
Klebsiella	**V36**	0	12	27	9	0	16	16	30	0	6	15	0	0	0	24	13
Klebsiella	**C61**	0	14	23	0	0	12	9	29	0	0	15	0	0	0	22	14
Klebsiella	**C63**	0	18	23	9	0	15	19	30	0	0	16	0	0	0	22	14
Klebsiella	**C64**	0	16	24	0	0	13	0	28	16	26	15	0	19	29	23	15
Klebsiella	**C67**	0	15	25	9	0	16	14	30	0	0	16	0	0	0	24	16

Table 6. Zone diameter sizes, mm, for village and campus isolates, organised by 16S sequencing result.

16S	ID	AMP	AMC	FOX	CTX	CPD	CFQ	ATM	IPM	S10	OT	CIP	NA	SXT	C	F	AZM
Klebsiella	**C69**	0	0	24	9	0	15	15	28	0	0	16	0	0	0	22	14
Stenotrophomonas	**V25**	0	14	0	14	0	23	0	0	16	24	29	20	22	26	0	19
Stenotrophomonas	**V28**	0	0	0	18	0	22	18	0	19	23	28	21	32	22	0	25
Stenotrophomonas	**V29**	0	21	21	13	0	25	23	0	14	23	22	24	28	23	0	21
Stenotrophomonas	**V31**	13	18	13	18	0	26	14	0	0	24	21	19	23	20	0	16
Stenotrophomonas	**V32**	26	40	29	29	0	34	22	26	20	28	18	25	34	24	0	11
Stenotrophomonas	**V33**	0	16	13	0	0	12	0	16	12	19	22	19	21	20	0	16
Stenotrophomonas	**V34**	0	8	0	20	0	20	16	0	17	21	27	21	19	26	0	21
Stenotrophomonas	**C59**	0	32	20	13	0	23	0	21	17	24	19	22	29	27	0	9
Stenotrophomonas	**C62**	18	16	0	28	0	34	32	0	7	21	23	22	30	26	0	15
Stenotrophomonas	**C65**	9	34	24	21	0	29	0	23	16	26	18	28	30	29	0	10
Stenotrophomonas	**C66**	0	12	0	19	0	23	25	0	16	18	22	16	22	19	0	17
Acinetobacter	**V35**	20	25	26	26	20	32	25	35	0	29	19	9	20	20	12	28
Acinetobacter	**C68**	17	31	25	25	19	31	23	37	9	26	21	0	20	22	22	23
Acinetobacter	**C70**	19	22	25	26	20	32	25	38	7	28	20	0	22	21	12	26
Unknown	**C56**	0	11	7	29	24	30	30	20	11	11	19	11	20	31	23	20
Unknown	**C57**	0	11	7	30	25	31	32	22	11	11	22	10	21	31	26	21
Unknown	**C58**	0	11	7	30	26	31	30	21	12	11	20	11	21	32	25	21