The action of metal cations on antimicrobial breakpoints in veterinary isolates of *Escherichia coli* and *Pseudomonas* spp.

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Contents

Abstract

Antibiotic use and misuse since their introduction have led to increasing bacterial resistance which coupled with the lack of development of new antimicrobials poses a global threat to both human and veterinary treatments. As a result of antibiotic resistance, treating bacterial infections in human and agricultural populations has become more difficult.

A gold standard method for testing antimicrobial susceptibility of clinical isolates was adopted within the UK in the 1950s with the current standard recommended by EUCAST. However, no set guidance is available for testing agricultural isolates with very few antibiotic breakpoints available.

The overall aim of this study was to identify whether certain metal cations within testing media affects the susceptibility of clinical and agricultural *Pseudomonas aeruginosa* and *Escherichia coli* isolates towards 4 panels of antimicrobials and to determine if this could lead to over or under reporting the efficacy of antimicrobials.

A representative sample of bovine gut (BG) and environmental (ENV) isolates from previous research at Kingston University were selected for use within this study, totalling 113 bacterial isolates. These had been previously identified as *P. aeruginosa* (14 BG and 17 ENV) and *E. coli* (63 BG and 19 ENV) with their antimicrobial resistance profiles confirmed. High tetracycline and β-lactam resistance was observed in the initial study, with multidrug resistance seen in many of the isolates. Following confirmational testing of antimicrobial susceptibility, A proportion of these isolates (N=40) distributed between both species was redetermined using the EUCAST method and changes in antimicrobial susceptibility profiles previously noted in 20 of the isolates selected for progression in this project identified.

Increases in antimicrobial susceptibility was seen in 6 of 10 *E. coli isolates* (4 BG

and 2 ENV) and 8 of 10 *P. aeruginosa* (3 BG and 5 ENV) isolates now displaying sensitivity towards at least one antimicrobial following antimicrobial susceptibility testing. An attempt to re-establish initial antimicrobial susceptibility profiles through double exposure towards an antimicrobial was achieved with β-lactam and tetracycline resistance seen to return in the environmental isolates of *E. coli* (n=3) and *P. aeruginosa* (n=3).

The EUCAST gold standard antimicrobial susceptibility testing method utilises media which contains trace amounts cations to allow for homeostasis within the bacterial cultures. The concentration of cations varies from manufacturer which has been seen to affect the susceptibility of antimicrobials on isolates (Girardello *et al*., 2012). Cations may be seen as a contributing factor to antimicrobial resistance with their ability to alter ion gradients within and around the cell affecting cell surface charges and interactions with protein channels like efflux pumps which are present in prokaryotes as well as interacting with antimicrobials contributing to development of antimicrobial resistance.

Efflux pump inhibitors are used to target efflux pumps within prokaryotes and have been studied in an attempt to combat antimicrobial resistance with several modes of actions proposed. The main focus of efflux pump inhibitors are as potential therapeutic agents to rejuvenate antimicrobials which have lost activity. The efflux pump inhibitor Phenylalanine arginine β-naphthylamide (PAβN) was selected as a well-studied inhibitor and a target for the resistant nodule division efflux pumps which have a strong prevalence in both *E. coli* and *P. aeruginosa.* A final concentration of 10 µg/ml was utilised in this study to identify changes in minimum inhibitory

concentration of: Cefoxitin, Ceftazidime, Cefepime, Ciprofloxacin, Imipenem, Amoxicillin, Amoxicillin+ Clavulanic acid and Tetracycline. Some changes in the minimum inhibitory concentration of ceftazidime against *P. aeruginosa* bovine gut isolates with one isolate showing a decrease from 1 µg/mL on Mueller-Hinton agar to 0.5 µg/mL with the cation adjusted media and with Phenylalanine arginine β-naphthylamide. A different isolate showing a decrease with the presence of the efflux pump inhibitor and cation adjusted media from 6 µg/mL to 4 µg/mL towards cefepime. A decrease in minimum inhibitory concentration for cefepime was also observed in an *E. coli* environmental isolate with the combination efflux pump inhibitor and cation adjusted media with a decrease against normal Mueller-Hinton agar from 0.125 µg/mL to 0.064 µg/mL.

The preliminary results indicate that changes in antimicrobial susceptibility testing conditions like increased concentration of cations and the presence of efflux pump inhibitors can affect the susceptibility of isolates towards antimicrobials and lead to changes in reporting of isolate susceptibility. However, these were initial observations and further testing is required with various concentrations of Phenylalanine arginine β-naphthylamide and cations present within the media to confirm these results on veterinary isolates.

Abbreviations

Abbreviation	Definition
ABC	ATP Binding Cassette
AMR	Antimicrobial Resistance
AST	Antbiotic Susceptibility testing
ATP	Adenosine Triphosphate
BG	Bovine Gut
BSAC	British Society for Antimicrobial Chemotherapy
CFU/ml	Colony Forming Units per milliliter
CLED	Cystine-Lactose-Electrolyte-Deficient Agar
CLSI	Clinical and Laboratory Standards Institute
DDD	Defined Daily Dose
ENV	Environmental
EPI	Efflux Pump Inhibitor
ESBL	Extended Spectrum Beta Lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agriculture Organization
MAC	MacConkey Agar
MATE	Multidrug And Toxic Compound Extrusion
MDR	Multidrug Resistance
MFS	Major Facilitator Superfamily
MHA	Mueller-Hinton Agar
MHB	Mueller-Hinton Broth
MHCA	Mueller-Hinton Cation Adjusted Agar
MHCB	Mueller-Hinton Cation Adjusted Broth
MIC	Minimum Inhibitory Concentration
NA	Nutrient Agar
NaPyr	Sodium Pyruvate
NB	Nutrient Broth
OHC	One Health Concept
OIE	World Organisation For Animal Health
PAβN	Phenylalanine Arginine β-naphthylamide
PBP	Penicillin Binding Protein
PPE	Personal Protective Equipment
RND	Resistance Nodule Division
SDW	Sterile Distilled Water
SMR	Small Multidrug Resistance
UTI	Urinary Tract Infection
WHO	World Health Organization
ZOI	Zone of Inhibition

1. Introduction

1.1 History of infection and infection control

Antimicrobial resistance predates our clinical discovery with low levels of tetracycline detected in Nubian bones dating back 2000 years (Bassett *et al*., 1980, D'Costa *et al*., 2011). Attempts to control the spread of infection has been around for many centuries with quarantining patients with leprosy and the plague with reference in various scientific and religious texts (World Health Organisation 2007). Before the identification of the infectious agents and development of synthetic compounds, the use of herbal and natural remedies was common practice dating back to ancient Egypt (Torrence and Isaacson, 2008; Gould, 2016)

The rise in awareness and development of germ theory in the early 19th century by Ignaz Semmelweis and Louis Pasteur (Faintuch and Faintuch, 2019) lead to the understanding and development of the idea of antisepsis (Crellin, 1981; Bednarek, *et al*., 2020). The subsequent development and use of antiseptics in the late 1800s coupled with the implementation of hand washing techniques, (Worboys, 2013) improved the morbidity and mortality rate within populations. At the beginning of the 20th century, life expectancy had increased by 10 years with men and women living to 58 and 64 respectively (Causes of death over 100 years - Office for National Statistics, 2017), in part due to improved nutrition, sanitation as well as disinfection interventions (Runcie, 2015).

Treatment of microbial infections in the beginning of the 20th century was named chemotherapy by Paul Ehrlich (DeVita and Chu, 2008) referring to the development of therapeutics by means of man-made chemicals (Schwerin *et al*., 2016). Ehrlich

determined that ‘the compound for treatment should contain a chemical group for fixation and another with selective toxicity which wouldn’t afflict humans’, in addition to being, ‘parasitrophic’ (Landecker, 2019; Liebenau, 1990). His compounds derived in the early 20th century were arsenic and mercury based and had many side effects such as kidney damage, seizures and fevers (Parascandola, 2009) with different outcomes *in vitro* and *in vivo* conditions varied with toxicity of the compounds shown to both humans and microorganisms (Landecker, 2019).

However, bacterial infections were not only seen in humans but also agricultural livestock, like Bovine tuberculosis causing around 3000 deaths worldwide in humans annually from contaminated milk (Woods, 2011). One of the few treatment options used for controlling many different infections in livestock was slaughtering. This was utilised in treating bovine Brucellosis and swine fever initially (Hope and Vordermeier, 2005; Dorneles *et al*., 2015).

1.2. History of antibiotics and usage

Before antibiotics, bacteriophages were used in treating dysentery in the 1900s by Félix D’Herelle in Paris. Bacteriophages were defined as a virus which affects and replicates only within bacterial cells (Kasman, 2020). At this time, the potential integration of viral DNA into the bacterial chromosomes was unknown (lysogeny) and as such therapy success was mixed. As a result of this and the discovery and availability of antibiotics, phage therapy was abandoned by many by the mid-twentieth century (Summers, 2012).

The first antibiotic was pyocyanase, a yellowy green substance secreted by *Pseudomonas aeruginosa* thought to have enzymatic properties was used to treat nosocomial infections (Aminov, 2010). Pyocyanase was injected into patients in order to suppress other pathogens like such causing anthrax, cholera and diphtheria (Hays *et al*., 1945; Caltrider, 1967). This was abandoned due to inconsistent results and limited success in treatment coupled with instability of the compound and toxicity towards humans (Aminov, 2010).

The first mass produced antibiotic was penicillin discovered by Sir Alexander Fleming in 1928 by accident with the growth of *Penicillium notatum* mould on an open plate of *Staphylococcus aureus* left out while away. Initial attempts to replicate the production of penicillin on larger scales were unsuccessful but achieved by Florey and Chain in 1941 (Gaynes, 2017). The mass production of penicillin decreased mortality caused by infections from World War 2 (Williams, 2009), However the leading cause of mortality was still infections caused by both Gram-positive and Gram-negative species like pseudomonads and *Clostridium* spp. (Eardley *et al*., 2011). The discovery of streptomycin followed shortly in 1944 and so began the golden age of antibiotics. Current antibiotics in use today were developed during the peak of discovery of antibiotics in the 1960s (Gould, 2016). However, the development of antibiotics in the 1980s dried up, and an inverse relationship to that with antibiotic resistance was observed (Figure 1).

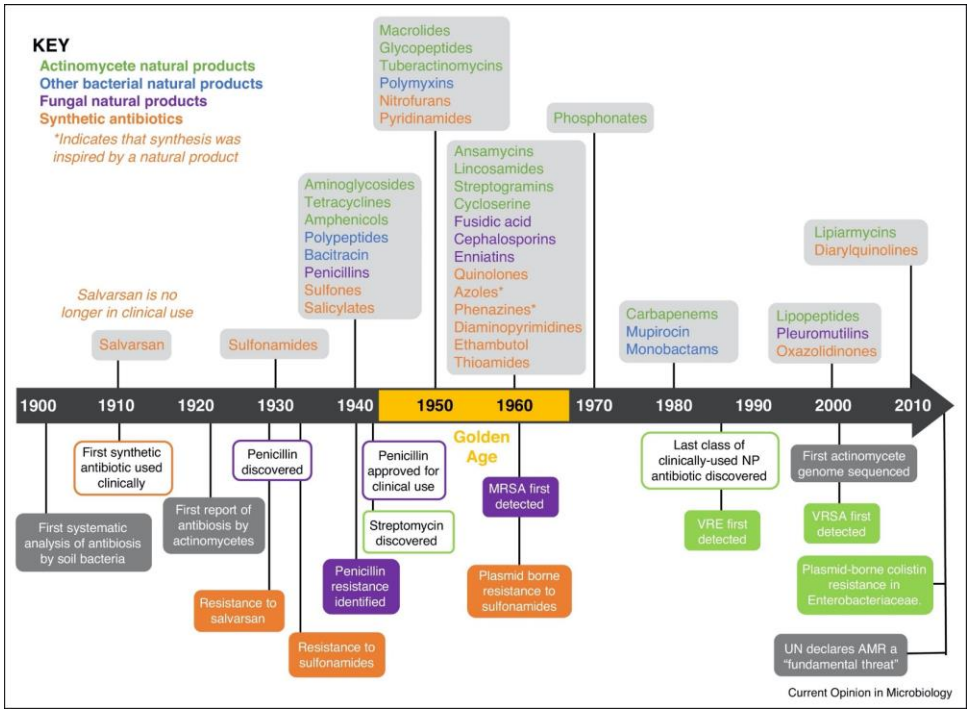


Figure 1: Timeline of the discovery and development of antimicrobials coloured by source of origin (Blue= other bacteria, Green= actinomycetes, Purple= Fungal and Orange= synthetic) with the bottom of the timeline showing the subsequent rise of resistance towards the antimicrobial. (Image taken from Hutchings *et al.*, 2019)

The deceleration of antibiotic development can be attributed to multiple factors with one major setback being financial, with reduced profitability due to the long-term investment as well as extended time frame needed for putting new antimicrobials into circulation. This reduced return is evident with only 1.6% of drugs in development in 2004 from 15 major pharmaceutical manufacturers being antibiotics (Jabes, 2020). There have been limited classes of antibiotics developed since the 1980s such as oxazolidinones and diarylquinolines being one of the last (Butler *et al*., 2013).

Human therapeutics

Since their introduction, antibiotics have been used to treat bacterial infections in humans with antimicrobial usage peaking at 531.2 metric tonnes in the UK between 2010 and 2013 (HM government, 2013). Unnecessary and inappropriate use

coupled with the lack of development of new antibiotics has led to an increase in antimicrobial resistance (AMR) and the development of multidrug resistant (MDR) bacteria (Ayukekbong *et al*., 2017), threatening our ability to combat once seemingly insignificant infections such as Urinary tract infections (UTI) caused by a patient's flora. Between 2004 to 2014 the number of diagnosed UTIs based on prescribing patterns was 21% of patients who matched the clinical criteria for UTI's (Ahmed *et al*., 2018). Of the tested urine samples to confirm diagnosis, 34% showed resistance towards trimethoprim with an increase of 4.9% compared to 2015 figures showing an increase in antibiotic resistance (Public Health England, 2016).

Between 2013 and 2017 the number of antibiotics used in the UK rose to 773 tonnes with 64% used in human medicine with 80% used in community care (Veterinary Medicines Directorate 2019). The use of antibiotics in human medicine between 2009-2018 showed an overall decrease of 1.6% of total consumption (European Centre for Disease Prevention and Control, 2019).

Veterinary medicine

Although primarily identified for human therapeutics, antibiotics were found to have alternative uses not only for treating infections in livestock but as growth promotors (Butaye *et al*., 2003). The production of cheap protein following World War 2 resulted in the use of antimicrobials as growth promotors in low doses administered to livestock (Jutzi, 2004). This use was discovered accidentally with the use vitamin B derived from fermented tetracyclines to promote growth in chicks, however this effect was later discovered to be due to the subtherapeutic doses of tetracycline received and not the fermented vitamin B12 (cobalamin) (Harremoës *et al*, 2002). However, it

did promote muscle growth and reduce subclinical infection in populations and was widely adopted as a result.

Antibiotics were previously used prophylactically in livestock for prevention of infection like bovine mastitis with transmission to calves through colostrum during suckling (Abb-Schwedler *et al*., 2014; Suojala *et al*., 2013).

Post 2000 we have felt the repercussions from prophylactic antibiotic treatment within the food chain, with leaching of antibiotics into the environment through water supplies leading to a rise in AMR in wild bacterial strains. (Manyi-Loh *et al*., 2018; Kivits *et al*., 2018; Cycoń *et al*., 2019).

The ban on using antibiotics as growth promotors in the UK was introduced in 2006 by an EU directive. The rise in AMR in food producing livestock can be seen to affect the use of antibiotics in human therapeutics as shown by the study in Vietnam and Thailand whereby increase in MDR towards fluoroquinolones of *Salmonella* spp. and *Campylobacter* spp. was seen in slaughtered livestock. This led to changes in treatment using alternative classes of antibiotics to those which were consumed by humans through contaminate meat (Padungtod *et al*., 2008). A similar observation was seen in the study in China by Liu *et al* (2015) whereby increase in resistance to colistin and increase in carbapenem resistance in food producing animals and subsequently in humans.

Current use of antibiotics in veterinary medicine is set out in the 15 recommendations within the One Health report and UK AMR vision with control and conservation key (Veterinary Medicines Directorate., 2019). This included a ban on growth promotors in livestock, prescribing in accordance with risk of resistance and justification for out of license use in addition to rapid diagnostics in food producing animals (Kirchhelle, 2018).

1.3 Emergence of antibiotic resistance

1.3.1 History and background

The first notation of antibiotic resistance was observed initially by Sir Alexander Fleming not long after the mass production and distribution of penicillin in the 1940s. During the late 1940s the production of penicillinase was observed in *E. coli* in addition to being observed in multiple strains of *S. aureus* in 1942 (Lobanovska and Pilla, 2017). Although this phenomenon started towards the end of the golden age of antibiotic discovery, the end of the 1960s, the rise in antibiotic resistance has been significant in the last decade with MDR being seen as posing an imminent threat on human and veterinary therapeutics. The World Health organisation (WHO) developed a One Health Concept in 2009 as an international collaboration in an attempt to combat antimicrobial resistance.

1.3.2 Development and overcoming of antibiotic resistance in prokaryotes

Resistance to antibiotics has been seen to predate the human discovery and clinical use of antibiotics with genes holding β-lactam and tetracycline resistance found in sediment from 30,000 years ago (D'Costa *et al*, 2011). One of the first observations of the development of antimicrobial resistance was in the 1950s after the introduction of penicillin and seen in Staphylococci through the production of penicillinase. This prompted further development by synthetic alterations to the penicillin ring structure resulting in several different β-lactam generations, known as semi-synthetic penicillins such as amoxicillin and ampicillin which have helped to preserve the class of penicillin antibiotic from becoming obsolete (Oshiro, 1999). Following the discovery of penicillin, Abraham and Chain classified penicillin binding proteins (PBP) including β-lactamases, grouping them into 17 functional groups which confer

resistance towards penicillin (Bush, 2018). Cross-species infections are defined as pathogens which have the ability to cause zoonotic and human infections from the same pathogen with *Pseudomonas aeruginosa* and *Escherichia coli* being examples of this (Tan, 2002).

Acquisition of antimicrobial resistance can occur various ways. One mechanism is mutation via acquisition or deletion of nucleotides causing phenotypical changes and mutations. The mutations that arise can change the action of antibiotics within the cell by decreasing the affinity of the drug, increase in efflux mechanisms, decrease in the drug uptake and modulation of various internal metabolic pathways;

- Limiting drug influx is seen in Gram-negative bacteria with the lipo-polysaccharide layer acting as a barrier preventing antibiotic access. The number of porins present on the surface of bacteria can limit access points for the antibiotic entry, which has been demonstrated with the reduction of porins in Enterobacteriales which has been observed to contribute to carbapenem resistance (Cornaglia *et al*., 1996; Reygaert, 2018).
- Enzymatic alteration of antibiotics leading to drug inactivation, which has been observed with β-lactamases. β-lactamases can be seen to destroy the amide bond within β-lactam ring (Figure 2). Previous studies of β-lactamases and β-lactamase like proteins have been compared which have shown to have a predisposition to antimicrobial resistance prior to our early discoveries of antimicrobials in the 20th century. Currently over 1000 different penicillin binding proteins (PBP) have been discovered which are sensitive to penicillin and penicillin-based antibiotics (Bush and Jacoby, 2009; D'Costa *et al*., 2011). Therefore the number of PBP available to interact far outweighs the

development of β-lactam antimicrobials, giving an increased risk in MDR bacterial pathogens.

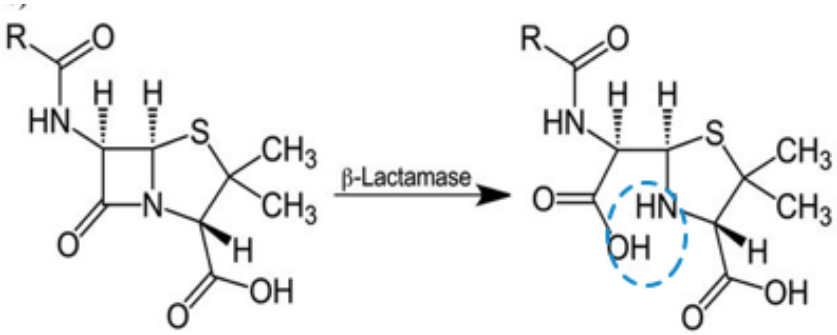


Figure 2: The cleavage of β-lactam ring of a penicillin antibiotic through interaction with β-lactamase protein causing changes in antibiotic structure by hydrolysis and becoming inactive. The blue ring identifies the outcome of the hydrolysis and the break in the of β-lactam ring (Antiangina, 2014).

Biochemical have also been observed with resistance to tetracycline *in vivo* with TetO and TetM with competitive binding to dislodge the antimicrobial on the 30s ribosomal unit (Wilson, 2013). Many of the antimicrobial modifications are done by catalysing the harbouring enzymes on mobile genetic elements such as plasmids whereby the resistance genes can be transferred, however aminoglycoside modifying enzymes can also be found coded within the chromosome in some bacterial species. The aim is to decrease the binding of the antibiotic towards its target causing an increase in susceptibility (Munita and Arias, 2016).

Resistance towards β-lactams has been combatted with supplementing with broad spectrum enzyme inhibitors like clavulanic acid being utilised in both community and nosocomial setting. A prime example of this is the increase in resistance to tobramycin in *P. aeruginosa* whereby reduced susceptibility was seen however this was overcome with cations where an increase in penetration of tobramycin was observed (Hall and Mah, 2017).

- The final mechanism is drug efflux which utilises various different efflux pumps which are present in prokaryotes and have multiple functions, which include the removal of a variety of compounds out of the cell (Cornaglia *et al*., 1996, Soto, 2013, Ambudkar *et al*., 1999, Paulsen, 2003). Efflux pumps have a high prevalence in MDR infections in both human and veterinary isolates with them become a target for AMR. Efflux pumps have been identified in both Gram-positive and Gram-negative isolates with their ability to cross-infect species and carry MDR (Davin-Regli *et* al., 2021).

Random genetic mutations can contribute to antibiotic resistance such as, causing different interactions within the cell like changes in efflux pump expressions or production of different enzymes and antimicrobial interactions. Mutations occurring in the active sties of class B β-lactamases can lead to changes in specificity resulting in the hydrolysis of larger molecules such as cephalosporins (Egorov, *et al*., 2018). Another mechanism by which resistance can develop is gene transfer which is acquisition of foreign DNA from their surrounding environments by one of the following possible processes. This can be done by vertical gene transfer during binary fission whereby random mutations can occur within the chromosome or plasmid during bacterial cell replication or horizontal gene transfer (HGT) by:

1) Transformation which is naturally occurring uptake of free genetic material from within the environment using homologous recombination. Transformation utilises single stranded DNA fragments transported through secretory pores into the periplasm within Gram-negative bacteria (Bennett, 2009, (Juan *et al*., 2015).

2) Transduction requires bacteriophages to encapsulate host genetic material during replication with ejection of bacterial DNA. Uptake of the ejected

bacterial DNA can occur and recombination within the new hosts chromosomes or plasmid (Bennett, 2009).

3) Conjugation requiring cell to cell contact with plasmid transfer from donor F+ to F- recipient across the pilus. With HGT the main rise of MDR in nosocomial infections is done by conjugation with direct cell to cell contact seen with Gastro-intestinal infections. The presence of conjugative plasmids in bacterial cells holds both resistance genes previously present form mutations or replication lineage and acquired resistance genes from horizontal gene transfer, (Bennett, 2009).

Human therapeutics

Nosocomial infections with improper treatment caused by *P. aeruginosa* has led to development of MDR species which can cause infection in minor burns and wounds to life threatening leading to death (Davies and Davies, 2010). *E. coli* can cause severe infections starting from simple UTI to complex bacteraemia with various strains able to live symbiotically and cause different infections in both humans and animals.

Veterinary infection

Within the agricultural sector, exposure of antimicrobials can come from various sources like feeding troughs and fields whereby antimicrobial leaching into the ground or washed as surface run, contaminated water supplies and underground reservoirs in addition to supplementation. The prolonged exposure of low dose antimicrobials in agriculture has shown to contribute to the development of AMR and MDR bacterial isolates present in livestock. This can lead to a higher likelihood of resistance transmission to and from the biosphere (Fernandes, 2003).

The UK has used a collective of various approaches in the battle against AMR like, identifying resistance breakers, discovering novel compounds, and repurposing current drugs in the market. In addition, there have been a number of compounds being tested over the last decade that permeabilise the outer membranes and anti-virulence compounds such as cationic peptides and compounds that will disrupt the production and formation of biofilms (Annunziato, 2019).

Biofilms

Both *P. aeruginosa* and *E. coli* can be observed in both planktonic and biofilm states. *E. coli* can be seen as part of a biofilms within the natural gut flora (Ishikawa *et al*., 2020). Biofilms form in 5 stages with the to end stage quorum sensing allowing for extracellular signalling and genetic expression which can attribute to AMR (Garrett *et al*., 2008). This allows for bacterial cells containing plasmids with resistance genes to be transferred through the biofilm by horizontal gene transmission conferring antimicrobial resistance which can spread through the formation separate colonies and biofilm expansion (Thanner *et al*., 2016; Lindow and Brandl, 2003).

The biofilm provides protection from not only environmental factors such as pH, temperature, disinfectants and antimicrobials as well as immune responses from the host. Variables are introduced within growth curve calculations for biofilms due to their structure (Verotta *et al*., 2017). The presence of biofilms accounts for around 80% of multiple chronic infections due to their ability to decrease susceptibility towards antimicrobials in comparison to planktonic cells (Sharma *et al*., 2019).

1.4 Antibiotic classification

Antibiotics of critical importance

Due to the limited discovery of new antibiotics over the past 3 decades the WHO produced a list categorising each antibiotics by importance and compared against several criteria internationally with regular reviews of the list. The 6th revision of the list of antimicrobials occurred in 2018 which classified 35 antimicrobial agents with further breakdown of antimicrobials within each class. The classification is initially based on acquisition of resistance genes from non-human sources and infections transmitted via non-human sources or sole or limited available therapy for infection in human medicine (World Health Organization, 2019).

Antibiotic classification in veterinary medicine

The OIE produced a criterion to classify each antibiotic class in regard to their veterinary importance. Each antibiotic class and agent are compared to several criterion and placed into one of 3 categories; Critically Important Antimicrobial Agents, Highly Important Antimicrobial Agents and Important Antimicrobial Agents. This gives classification to all the current antibiotic and antimicrobial compounds available for use in veterinary medicine with the majority of antibiotic classes showing as critically important (World Organisation for Animal Health, 2019).

1.5 Antibiotic Susceptibility testing

The observation of inhibition of bacterial growth by Sir Alexander Flemming led to the accidental discovery of penicillin through inhibition *S. aureus*. Following this observation various methods have been developed for antibiotic susceptibility testing (AST) over the past 4 decades. The phenotypic methods include but are not limited to antimicrobial disc diffusion, Microbroth dilution, E-test (Khan *et al*., 2019; Balouiri *et al*., 2016).

The Kirby-Bauer Method utilises sterile discs which are impregnated with known concentrations of antimicrobials placed onto inoculated agar plates prior to incubation (Bauer *et al.*, 1966). Following incubation, the Zones of Inhibition (ZOI) are read to the following day. The Kirby-Bauer method was confirmed to be the international gold standard testing method in 1956 by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), Clinical and Laboratory Standards Institute (CLSI) and is used in both human and veterinary medicine (Khan *et al*., 2019).

One of the first record of broth dilution AST was in the 1870s (Balouiri, *et al*., 2016; Khan *et al*., 2019), involving two-fold dilutions of antimicrobial agent and liquid growth medium, with changes in visual turbidity through different concentrations. Visual turbidity or plate readers are utilised in determining concentration for inhibition.

The E-TEST method holds similar principles to the disc diffusion method apart from the impregnated test strips contain a range concentration rather than a single concentration (Bolstrom *et al,.* 1988). The ZOI is read the following day like the disc diffusion method, However the Minimum inhibitory concentration (MIC) is the point of intersection on the E-TEST strip (Khan *et al*., 2019).

1.5.1 Testing Human isolates

EUCAST was formed from the European Society of Clinical Microbiology and Infectious Diseases in 1997 to create a committee of standardisation similar to those such as the CLSI and the British Society for Antimicrobial Chemotherapy (BSAC). Prior to standardisation, many laboratories used or adapted the method from CLSI. Regular consultation with various industries, including pharmaceutical and manufacturing, as open access consultations were performed to allow for integration within the decision process of the EUCAST committee and sub-committee (Kahlmeter, 2015).

The standardisation came about in 2007 (Matuschek *et al*., 2014) with the methodology designed by EUCAST utilising the Kirby-Bauer method. This is based on phenotypic AST with breakpoints based on the MIC value, which in turn has quality control against various bacterial strains routinely to allow for adjustments of breakpoints (Kahlmeter, 2015; Desmet *et al*., 2016) which is updated annually. (European Committee on Antimicrobial Susceptibility Testing (EUCAST), 2020).

<u>Factors affecting AST</u>

There are several factors which can influence AST which can lead to skewed outcomes which in turn may influence non-optimal treatments. Changes in inoculum density can lead to variation of the ZOI with light inoculum giving false positives of sensitivity, thereby a standard for inter and intra-laboratory comparison was required. A 0.5 McFarland is used which gives an approximate 1-5 x 10^8 colony forming units per millilitre (CFU/ml) providing a standardized range for microbial testing (European Committee on Antimicrobial Susceptibility Testing, 2020). The time between application of antimicrobial impregnated discs and inoculation should be done within

15 minutes as recommended by EUCAST as to limit bacterial replication in addition to incubation temperature. Each species holds standardised incubation times of which over or under incubation can lead to unreliable reports of susceptibility. The conditions of incubation will also affect the growth of the organism such as anaerobic, aerobic and fastidious organisms requiring altered incubation conditions (Smaill, 2000).

The potency of impregnated discs may deteriorate over time in addition to incorrect storage which can cause changes in ZOI. The composition of testing media can also influence the ZOI in addition to growth of inoculum which can affect the diffusion of antimicrobial into the agar, thus a standardised depth of plate of 4mm +/- 0.5 mm is recommended by EUCAST, CLSI and BSACS for susceptibility testing. Both CLSI and EUCAST recommendation of Mueller-Hinton agar for susceptibility testing with additives used depending on the species being tested and hold recommendations for AST within their methodology (European Committee on Antimicrobial Susceptibility Testing, 2020; Clinical Laboratory Standards Institute. 2006). The incorrect use of Personal Protective Equipment and improper decontamination can lead to contamination of laboratory surfaces, cultures and isolates being tested from the natural skin flora or cross contamination (Nogueras *et al*., 2001; Genzen, 2020).

1.5.2 Susceptibility testing in veterinary isolates

The testing of isolates from veterinary origins does not rely on a single standardised method but several different methods. However as there is no complete set of antimicrobial breakpoints for veterinary isolates, this makes it more difficult when trying to define those causing disease in animals. Guidance for susceptibility testing

is available from CLSI with the VET01 2018 documentation which gives methodology towards micro-dilution and disc diffusion methods which are similar to EUCAST; However, this does not account for variation between animal species. The recommendation for veterinary testing by CLSI and EUCAST is the Kirby-Bauer method or using E-TESTS, both impregnated with antibiotics. The VETCAST subcommittee uses CLSI breakpoints however no publication has been released attaining isolates of veterinary origin (Denham, 2018; Dargatz *et al*., 2017). The OIE set out a list of standards in 2018 for AST in terrestrial animals of which has been adopted by 182 member countries. The report gives detail of storage of veterinary isolates in addition to susceptibility testing as well as reporting of resistance to WHO/ Food and Agriculture Organization. The three methods recognised as standard and adopted also include broth and agar microdilutions ((World Organization for Animal Health, 2015)).

1.6 Metal Cations

Various cations are present in both eukaryotes and prokaryotes with their presence as monovalent or divalent depending on their electrochemical charge. Cations Like H^+ play an imperative role in cellular regulation and function and are utilised for metabolic processes in both eukaryotes and prokaryotes (Baykov *et al.*, 2013). The function in both is very similar allowing for survival of cells and function of the organism by interaction with Adenosine triphosphate, ribosomal interaction, cell motility and division and maintenance of cellular membranes (Sigel and Sigel, 1996; Domínguez *et al*., 2015).

Prokaryotes

Prokaryotes have been shown to use various cations on a cellular and intracellular level, with cations such as Sodium (Na^+), Magnesium (Mg^{2+}) and Calcium (Ca^{2+}) playing an important role in efflux mechanisms (Silver, 1983). *E. coli* and the OmpF channel (Benz, 2006) has cationic variation and specificity when changes in surface charge on the pore walls occur. *E. coli* requires calcium ions to increase the affinity of binding of cobalamin transport across the membrane, in addition the presence of magnesium within the cytosol of which is used in the activation of extracellular enzymes (Lynn and Rosen, 1987, Romani, 2011). DNA replication in prokaryotes use topoisomerases also require the addition of metal cations such as Mg^{2+} as well as ATP to aid the uncoiling of the supercoiled DNA (Sissi and Palumbo, 2009). Hydrogen or H^+ ions play a vital role in the proton gradient which is used in multiple process within cells like interactions with ATP synthase for catalysing the formation of ATP from ADP (Neupane *et al.*, 2019) and proton motor force for cellular growth and maintenance (Wang et al., 2021) to efflux pump function through proton gradients for activation and exchange across the membrane (Pathania *et al.,* 2019).

The Gouy-Chapman theory which holds base principles for ion interaction and spacial distribution of higher positive ionic charges surrounding bacterial cells (Figure 3) due to their increased negative internal charge due to the presence of peptidoglycan (Silhavy., 2010). This allows for cationic interactions within the double layer and interactions to the bacterial cell (Yi *et al.*, 2008). This can be applied as base principles for cationic charges on bacterial cells however, the principle only holds for divalent cations and above as physiochemical properties need to be accounted for (Benz, 2006).

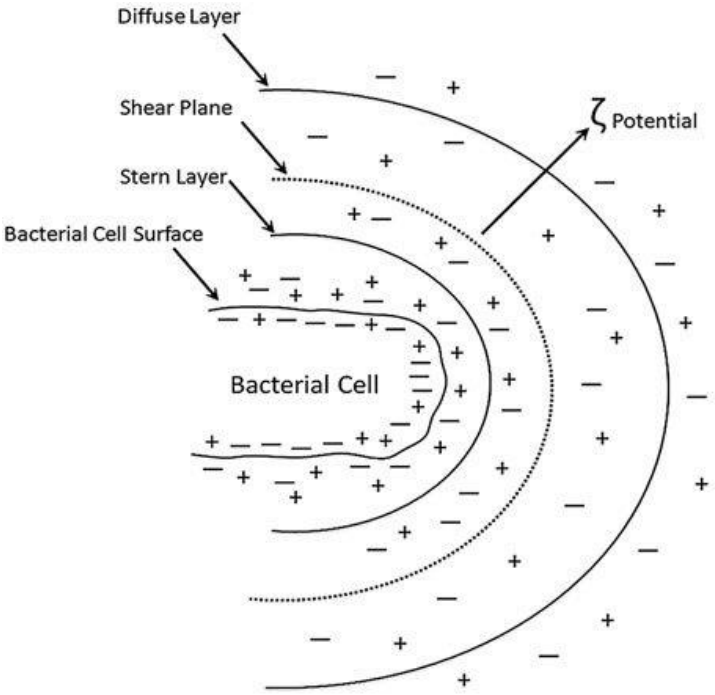


Figure 3: Schematic Representation of variation and spatial electrical charges around a generalised planktonic bacterial cell and ion gradient. This holds for negatively charged potentials allowing for cationic interactions (Pouran, 2017). Bacterial cell holds a greater negative charge due to the increased peptidoglycan presence causing a greater negative charge in the surround area or planes. This allows for positively charged ions such to enter into the bacterial cell. Stern layer: opposite charged ions near the bacterial cell. Shear plane: interface between diffuse layer and stern layer holding electrical potential. Diffuse layer: counter ion layer to hold and balance charges. Stern layer and diffuse layer are commonly referred to as the double layer (image taken from, Silhavy., 2010).

1.6.1 Interactions with antibiotics

Cationic interactions on protein channels within eukaryotes and prokaryotes have a profound effect on antibiotics susceptibility by action on efflux pumps and variation of membrane permeability. Each antibiotic and each class have different interactions with cations. The initial observation of interaction of cations was with fluoroquinolones in 1985 against *P. aeruginosa* which showed increased stability in the outer membrane and increased susceptibility towards ciprofloxacin (Höffken *et al*., 1985). The increase in susceptibility towards daptomycin was seen in Gram-positive bacteria using Mg^{2+} however stronger susceptibility was seen using K^{+} with increase in pores within the membrane (Zhang *et al*., 2014). Aminoglycosides have

showed antagonism with cations with increases in MIC such as gentamicin susceptibility testing against *P. aeruginosa.* The testing of *P. aeruginosa* was done on cation adjusted media for disc diffusion and micro-broth dilutions with both giving the same outcome (Barry *et al*., 1992).

Other studies have shown no significant difference in the MIC with the presence of cation adjusted media when susceptibility testing *E*. *coli*, suggesting that variation of susceptibility changes can vary by strain and species (Ramirez-Ronda *et al*., 1975). Due to the variation in β-lactamases and their dependency on cations such as zinc, decreases in susceptibility towards β-lactam antibiotics and carbapenems have been observed with mutations in the PBP (Papp-Wallace *et al*., 2011). Correlation between Chloramphenicol resistance and cations was established in the 1980s whereby the presence of Ca^{2+} and Mg^{2+} were seen to disrupt the structure, stability and function of the outer membrane of Gram-negative bacteria (Irvin and Ingram, 1982), with increase in antimicrobial susceptibility.

Tetracycline has a high affinity for divalent cations which aids in the accumulation within the periplasm where disassociation with the ion occurs to allow for tetracycline to diffuse through the pore into the cytoplasm increasing the antibiotics susceptibility (Chopra and Roberts, 2001).

Cations have also shown presence in microbiological media in trace amounts which vary between manufacturers as well as following sterilisation, with variation in concentration free divalent cations within the media being unpredictable like Biorad MH agar having 43 mg/L of Ca^{2+} yet Merck MH agar having 7.4 mg/L of Ca^{2+} ions (appendix 1) (Åhman et al., 2020, Murray and Zeitinger, 1983; D'Amato *et al*., 1975). With EUCAST not having a preferred brand for Mueller-Hinton media for

susceptibility testing it utilises several other bodies for media composition. However, it does set out that it should meet its quality control standards and meet the ISO standard (ISO standard 20776-1, 2016) in addition to guidance on media preparation and storage. Several studies have been undertaken to identify media composition and cation concentrations in several manufacturers Mueller-Hinton and Cation-adjusted Mueller-Hinton media as well as the recommended concentration by EUCAST (appendix 1). This shows that cation-adjusted media is within the range recommended for antibiotic susceptibility testing as defined in the EUCAST methodology.

1.7 Efflux Pumps

1.7.1 Structure and function

Efflux pumps play a vital role in cellular regulation like quorum sensing and removing substances such as antimicrobial agents, toxic substances and from the cytoplasm to the surrounding environment (Martinez et al., 2009, Soto, 2013). The first efflux pump identified in the 1970's was a p-glycoprotein which is ATP dependent and showed broad resistance to many chemotherapy agents (Ambudkar *et al*., 1999; Paulsen, 2003).

Efflux pumps are categorised into 5 families which are based on: the number of components required for the efflux pump, component functionality, the number of transmembrane spanning regions, the energy source required, and type of molecules transported (Figure 4).

Single component action efflux pumps on Gram-negative bacteria transfer molecules such as antimicrobials into the periplasmic space from the cytoplasm. Efflux Pumps can be coded on either transmissible plasmids and transposons or chromosomes.

The presence and expression of different families of efflux pumps varies between both Gram-positive and Gram-negative species with Gram-negative bacteria using resistance nodulation division efflux pumps primarily for drug efflux (Blanco *et al*., 2016).

Efflux pumps exist in one of two configurations; the first configuration is a transporter spanning cytoplasmic membrane whereby transport of substance to the external environment or periplasmic membrane occurs. The second configuration is a complex composition of efflux pumps and porins which are only present in Gram-negative bacteria utilising an assembly of different proteins (Figure 4) (Nikaido, 1998).

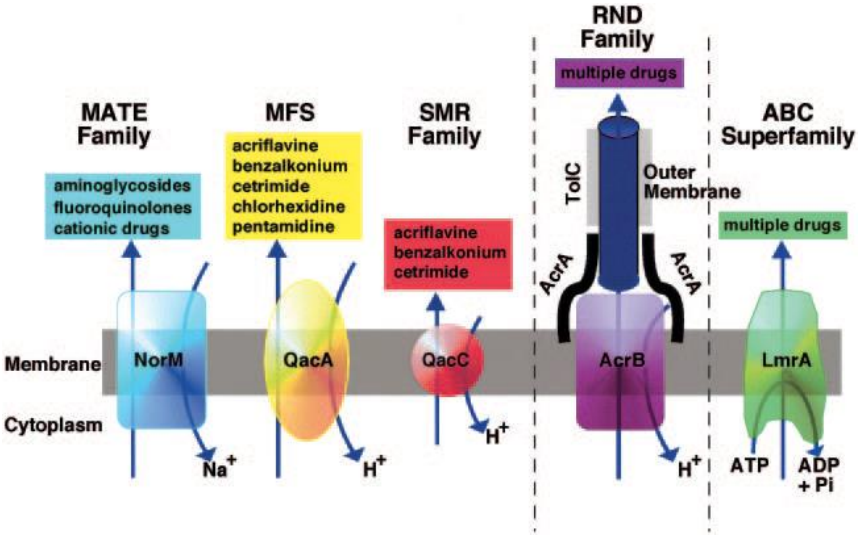


Figure 4: Representation of the 5 families and composition of efflux pumps in addition to their energy requirements (not to scale). RND efflux pumps identified as the largest efflux pumps compared to the other four families (Image taken from, Piddock, 2006).

1.7.2. Families of Efflux pumps

Resistance Nodulation Division (RND)

The RND family are tripartite efflux present in Gram-negative bacteria and used to transport multiple substances across the lipid membrane (Piddock, 2006) and plays a crucial role in MDR in clinical and wild pathogens (Morita *et al.*, 2012) which affect

humans and animals and their ability to form multiple efflux systems and interact various substrates (Mahamoud *et al*., 2007; Lee *et al*., 2000; Ruggerone *et al*, 2013). The MexAB-OprM system plays a role in MDR in *P. aeruginosa* (Aeschlimann, 2003) like the Arc-TolC family in *E. coli*. The composition of RND pumps require a transporter on the inner membrane, a periplasmic protein and an outer membrane protein channel and function as antiporters requiring a proton gradient and utilise one proton per molecule of drug exchange (Paulsen, 2003).

The over expression of RND Efflux pumps and its impact on MDR is seen in *E. coli* with fluoroquinolone resistance (Li and Nikaido, 2009) with decrease in intracellular antimicrobial concentration as well as efflux transport of biocides and other antimicrobial agents (Nikaido and Pagès, 2012). RND pumps are over expressed and most are prevalent in Gram-negative bacteria (Ruggerone *et al*., 2013) which play a crucial role in acquired and intrinsic antimicrobial resistance and affect virulence and colonisation (McNeil *et al*., 2019), thus making RND efflux pumps an ideal target for inhibition.

Small Multidrug Resistance (SMR)

The SMR family of efflux pumps are transmembrane across the cytoplasm and consist of 4 α-helices (Bay *et al*., 2008) and present *E. coli* (Bay and Turner, 2009). SMR are antiporters and are present in both Gram-positive and Gram-negative bacteria.

Major Facilitator Superfamily (MFS)

The MFS superfamily is one of largest families of efflux pumps which are seen in prokaryotes, eukaryotes and archaea and can function as solute uniport, solute and

cation symport and antiport, and multidirectional transport with dependency on polarity (Pao *et al*., 1998; Law *et al*., 2008). The presence of MFS has a high prevalence in *E. coli* with many MDR-MFS pumps like TetA which confers tetracycline resistance (Kumar *et al*., 2016).

Multidrug And Toxic Compound Extrusion (MATE)

MATE efflux pumps play a critical role in the acquisition of antimicrobial resistance in addition to intrinsic factors in many bacterial species and require an electrochemical gradient and cation dependency for drug transport. The MATE transporters have aided in the rise of MDR nosocomial infections with a high contribution towards fluoroquinolone resistance (Tanaka *et al*., 2013; Piddock, 2006; Kuroda and Tsuchiya, 2009).

ATP binding cassette (ABC)

The ABC family of efflux are responsible for the efflux of xenobiotics and metabolites (Pohl *et al*., 2011) and present in prokaryotes and eukaryotes (El-Awady *et al*, 2017). The ABC family is the largest protein transporter which consists of the transmembrane domain and the nucleotide binding domain (NBD) situated in the cytoplasm for direct ATP hydrolysis (Dawson and Locher, 2006; Choi and Yu, 2014). The presence of ABC efflux pumps can be seen on antimicrobial producing organisms to induce self-resistance like macrolide resistance (Marquez, 2005).

1.8 Efflux pump Inhibitors (EPI)

1.8.1 History of EPI

The categorization of efflux pump inhibitors (EPI) is based on the source of origin rather than mechanism of action. The presence and overexpression of RND efflux pumps in various prokaryotes contributes highly towards MDR (Mahamoud *et al*., 2007; Lee *et al*., 2000). PAβN was one of the first efflux pump inhibitors to be developed initially in 1999, with others following suit. PAβN was used to target *P. aeruginosa* to supplement the treatments using erythromycin and levofloxacin by acting on MEX AB-OprM system (Pathania *et al*., 2019). There are various known EPI such as Carbonylcyanidem-chlorophenylhydrazone (CCCP) which interfere with the electrochemical gradient, proton motor force, preventing efflux out of the cell and ultimately causing bacterial cell death due to accumulation of antimicrobials with the cells (Mahamoud *et al*., 2007). The inhibition of efflux pumps can occur in several ways from interference with regulation and composition of the efflux pump to competitive binding with substrates to increase accumulation within the cell in addition to interfering with the proton gradient however this is not the case with ABC efflux pumps which use direct ATP hydrolysis (Askoura, *et al*., 2011).

1.8.2 Phenylalanine arginine β-naphthylamide

Structure and function

PAβN belongs to a family called peptidomimetics which consist of a dipeptide-amide (Figure 5) and holds a low molecular weight allowing it to enter bacterial cells trough porins (Lamut, *et al*., 2019). There are two possible modes of action the first uses PAβN; prevent the movement of g-loop which in ATP binding in substrate transport from distal to proximal binding sites. The second is competitive binding of PAβN in

which conformational changes occur, which is the greater accepted theory of mechanism of action. Competitive binding of the efflux pump inhibitors leads to changes in substrate extrusion channel preventing expulsion of larger molecules such as antibiotics (Opperman and Nguyen, 2015).

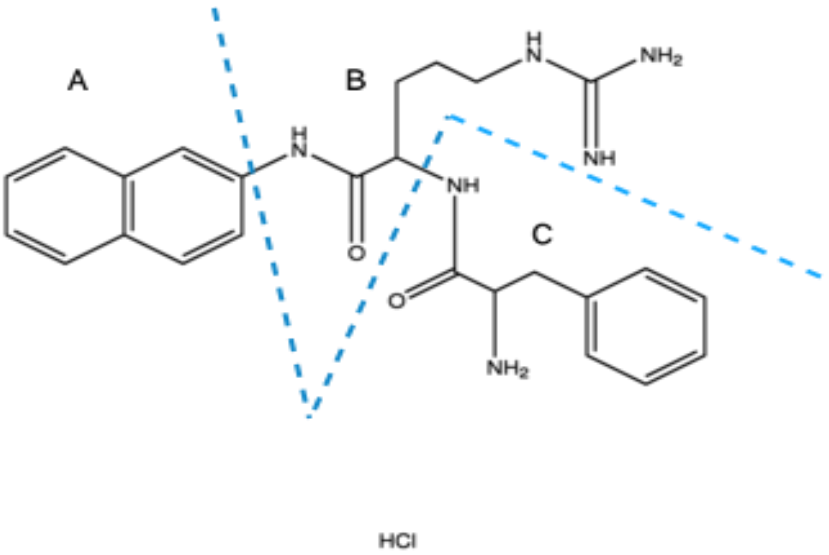


Figure 5: Chemical structure of PAβN. structure is divided (blue line) into 3 sections showing composition of the compound. A= Amide cap, B= Arginine, C=Phenylalanine (Opperman and Nguyen, 2015). Image adapted from Sigma Aldrich UK (Sigma Aldrich UK, 2020). Phenylalanine and arginine were identified as the more active and stable compared to their analogies during compound development. The amide cap selected of the analogues showed most antibacterial activity within the compound, thus this structure of analogues was selected as the first EPI target. Initial testing completed against *P. aeruginosa* (Renau *et al.*, 1999).

PAβN has been used alongside various antibiotics which have been used as an indicator for efflux pump activity (Vargiu *et al*., 2014). *In vitro* testing has previously shown the ability for the compound to restore susceptibility towards a range of antimicrobials like chloramphenicol and erythromycin (Vargiu *et al*., 2014). Since its development, most studies completed *in vitro*, looked at changes in Minimum Inhibitory Concentration (MIC) and determination of bacterial isolates with mutant in

efflux pumps with increased antimicrobial resistance such as *P. aeruginosa* with over expression of AmpC and Mex familial mutations (Lamers *et al*., 2013).

Effect on prokaryotes

PAβN primarily acts on Gram-negative bacteria due to the mode of action, with its ability permeabilizes the outer membrane (Lamers *et al*., 2013) acting on different efflux pumps without affecting the proton gradient (Gbian and Omri, 2021). With *P. aeruginosa* the primary family of efflux pumps conferring MDR are the RND family and the Mex family (Lamers *et al*., 2013; Lomovskaya and Watkins, 2001). PAβN acts as a competitive inhibitor for the RND efflux pumps, preventing antimicrobial binding and extrusion from the cell (Lamers, 2013; Piddock, 2006). The EPI has seen to increase susceptibility towards tetracycline (Amaral *et al*., 2014) and fluoroquinolones (Opperman and Nguyen, 2015) in *E. coli* and increase susceptibility towards levofloxacin in *P. aeruginosa* (Renau *et al.*, 1999, Opperman and Nguyen, 2015). Of the previous studies of MDR isolates over the past 20 years using PAβN, the observation are of similar nature with all showing both increase in susceptibility towards some classes of antimicrobials and its action on permeabilizing the outer membrane causing increased cellular toxicity.

1.9 Previous study of veterinary isolates

A previous research project at Kingston University in 2015 to 2016 involved the collection of samples from 3 dairy farms from 3 visits, over a period of 12 months. Samples were taken by veterinarians from the Bovine Gut (BG) and swabs from various environmental (ENV) locations within the farm prior to bacterial isolation and identification at Kingston University. The original research isolated 807 isolates of

which 59 were presumptively identified as *P. aeruginosa* and 361 as *E. coli* with the remainder of the isolates initially identified as *Streptococci* spp, *Klebsiella* spp, *Proteus* spp, *Salmonella* spp, *Staphylococcus* spp and *Corynebacterium* spp through biochemical testing, purity streaking, selective media and gram staining (Tuin, 2016).

The bacterial isolates were prepared, purified and bacterial species identified followed by AST and MIC determination in addition to calculating Epidemiological cut-off value. Percentages of isolates resistant to at least 1 antimicrobial from location determined by the previous KU student (Figure 6). From the previous KU students AST at least 50% of all the isolates identified for this project held resistance to at least 1 antimicrobial in addition to each farm having identified isolates whereby the selected isolates were resistant to at least 1 antimicrobial such as the *P. aeruginosa* BG isolates from farm 1 (Figure 6B) or the *E. coli* BG isolates selected from farm 3 (Figure 6D). The overall aim of the project was to determine the levels of antimicrobial resistance. Isolates that were identified and purified were stored at -80°C for further studies (Tuin, 2016).

Figure 6: A. Total number of *E. coli* and *P. aeruginosa* wild isolates identified and tested by the previous student (Left) in addition to distribution and number of the isolates initially selected for this project (Right). B-D shows the number of *E. coli* and *P. aeruginosa* isolates selected from each dairy farm with the percentages of isolates resistant to at least 1 antimicrobial. Data corresponds to left of the panel (Tuin, 2016).

1.10 Aim

The aim of the project is to identify whether the susceptibility of *E. coli* and *P. aeruginosa* clinical and veterinary isolates may be affected by the presence of metal cations in the presence of inhibitory substances which may lead to over or under reporting of the efficacy of the antimicrobials used in treatment.

This will be done by confirming the antibiotic sensitivity profiles of the isolates identified by the previous KU student through AST, followed by using alternate carbon sources and alternate media to observe bacterial growth, and finally analysing isolate susceptibility through MIC testing during exponential growth phase with and without the presence of cations and inhibitors.

2. Materials and Methods

2.1 Microbiological media

Nutrient agar (NA) (Sigma Aldrich UK) was prepared as per manufacturer guidelines using sterile deionised water and sterilised using an autoclave for 15 minutes at 121°C and 15 psi and aseptically decanted into sterile 90 mm Petri dishes (SLS Laboratories UK, Fisher Scientific UK) and allowed to set at room temperature. NA was dried by Laminar flow hood (Gelaire Australia) prior to use. Both Mueller-Hinton agar (MHA) and Mueller-Hinton cation adjusted agar (MHCA), (Sigma Aldrich, UK) were prepared as per manufacturing guidelines followed by sterilisation at 121°C and 15 psi for 15 minutes and aseptically decanted to a depth of 4 mm into 90 mm Petri dishes (SLS Laboratories UK, Fisher Scientific UK) and allowed to set. Mueller-Hinton broth (MHB) and Mueller-Hinton cation adjusted broth (MHCB) (Sigma Aldrich UK) were prepared as per manufacturing guidelines and 10 or 20 ml aliquots decanted into 25 ml glass universal bottles prior to sterilisation for 15 minutes at 121°C and 15 psi. Once sterilised and cooled broths were stored in a cool dry place for a maximum of 4 weeks. Compositions of the different Mueller-Hinton media used (Table 1).

Table 1: Composition of the different Mueller-Hinton media (OXOID Ltd, UK) utilised in antimicrobial susceptibility testing, cationic growth and MIC assays.

Media	Ingredients	Composition Gram/Litre
Mueller-Hinton agar	Beef infusion solids	2
	Starch	1.5
	Casein hydrolysate	17.5
	Agar	17
Mueller-Hinton cation adjusted agar	Beef heart infusion	2
	Starch (soluble)	1.5
	Acid Casein Hydrolysate	17.5
	Agar	17
Mueller-Hinton Broth	Beef infusion solids	2
	Starch	1.5
	Casein hydrolysate	17.5
Mueller-Hinton cation adjusted Broth	Beef extract	3
	Starch	1.5
	Acid Casein Hydrolysate	17.5

Although EUCAST doesn't have a preferred brand of Mueller-Hinton media they recommend that it should meet the requirement of ISO standard 20776-1, 2016 (appendix 1). For Non adjusted Mueller-Hinton and for cation adjusted Mueller-Hinton requires the same standard however has adjusted calcium and magnesium ions in the range of 20-25 mg/L; and 10-12.5 mg/L respectively. Difference in starch used in MHA and MHCA is seen (table 1) with MHCA using soluble, the manufacturer OXOID UK was contacted several times between august and December 2021 with no clarification given by the manufacturer. In addition, different meat infusion are used in the different MH and MHC with difference in source identified of dehydrated infusion from beef. No single source of beef extract is stated in the ISO recommendation with only the total quantity is 300g. the variation of acid casein and casein hydrolysate is seen as a result of meeting the (appendix 1).

Selective agars were used for identification of mixed cultures and purification of isolates; MacConkey agar (MAC) (OXOID Ltd) and Cystine-Lactose-Electrolyte-Deficient Agar (CLED) (Sigma Aldrich UK) was prepared as per manufacturer's guidelines and sterilised 15 minutes at 121°C and 15 psi. Agar was decanted aseptically to a depth of 4 mm and allowed to set. All agars were stored between 2°C to 8°C once set. All agar plates were dried using laminar flow hood (Gelaire Australia) prior to use.

MHA and MHCA with the addition of Phenylalanine-arginine β-naphthylamide (PaβN; Sigma Aldrich UK) was prepared as above however prior to aseptically decanting the agar, 5 mls of 1 mg/ml PaβN solution was added to molten agar to give a final concentration of 10 µg/ml. Agar was then decanted into 4 mm depth and allowed to set. Ringers Solution (OXOID, UK) was prepared by dissolving one tablet in 500 ml of sterile distilled water and decanted into 5ml and 10ml aliquots and was sterilised at 121°C and 15 psi for 15 minutes. Unused solution was discarded after 4 weeks.

2.2 Maintenance and growth of bacterial stocks

Original stocks of isolates

Original bacterial isolate stocks were divided by their farm and visit number. The environmental locations where samples were obtained were:

- Holding yard
- Scraper tractor tread
- Crush yard
- Feed passage

- Cubicle shed

Bovine gut samples were obtained by swabbing of veterinarian examination gloves following rectal examinations. Original stocks of isolates had been prepared, identified, purified and stored at -80°C by a previous student at Kingston University (KU) between 2015 to 2016. Random selected isolates were cultured onto NA and incubated for 24 hours at 37°C and passaged twice on solid media (NA) prior to use.

Isolate storage and maintenance of bacterial stocks

Overnight cultures of isolates were prepared onto NA from previous KU student stock and incubated at 37°C for 24 hours. From pure cultures, 5 single colonies were transferred into a Microbank vials (Prolabs diagnostic) and agitated. Inoculated vials were labelled appropriately and stored at -80°C.

Following storage of new isolate stocks at -80°C for 24 hours, a sample was taken aseptically using a sterile cotton swab and transferred onto NA and incubated at 37°C for 24 hours for confirmation of culture transfer to new stock.

2.3 Biochemical Testing

Various biochemical tests were utilized for identification of isolates where appropriate in mixed cultures. This included oxidase, catalase and urease tests. All biochemical tests were carried as control purposes on Methicillin-Resistant *S. aureus* NCTC 12493, *P. aeruginosa* NCTC 13359 and *E. coli* NCTC 11954.

Oxidase

A 1% w/v oxidase stock solution was prepared using Oxidase reagent (N, N,N′,N′-Tetramethyl-p-phenylenediamine dihydrochloride, TMPPD) (Sigma Aldrich UK) and sterilised deionised water into a sterile universal bottle which protects the content from direct light and stored between 2°C to 8°C. Solution was discarded within 24 hours.

Catalase

A 3% v/v solution of Hydrogen peroxide (H_2O_2) (Sigma Aldrich UK) was prepared using sterile deionised water and stored between 2°C to 8°C. The solution was added to sterile microscope slides and single colonies were added using a sterile plastic loop from overnight cultures and observed.

Urease test

Urea broth (Sigma Aldrich UK) was prepared as per manufacturing guidelines using sterile deionised water and filter sterilised using sterile syringes and 0.22 μm filters (Sigma Aldrich UK) into 5ml aliquots into sterile plastic bijous. Broth was inoculated with cells from an overnight culture aseptically and incubated for 24 hours at 37°C and observed the following day.

Gram staining

Microscope slide was flamed in alcohol to sterilise and remove any surface contaminants. A drop of sterile distilled water (SDW) was added to a microscope slide and inoculated with a single colony from overnight cultures. Sample were air

dried prior to fixing by flaming the reverse of the slide. The staining process was as followed:

- A 1% v/v Crystal violet solution was used to flood the microscope slide for 1 minute followed by rinsing off any excess solution using SDW.
- Lugols solution was then used to flood the inoculated microscope slide for a further minute followed by washing away excess stain using SDW.
- Inoculated slides were decolourised using (80:20) w/w ethanol/acetone then rinsed with SDW.
- The inoculated slides were flooded with carbol fuchsin and left for a further minute then rinsed again with SDW.

Stained slides were blot dried followed by observed under oil emersion light microscopy once dried at 100 magnification.

2.4 Antibiotic susceptibility testing (AST)

Antibiotic susceptibility testing was carried out using EUCAST methodology (Matuschek *et al*., 2014). Twenty antibiotics were utilized which had been identified by the previous Kingston University student (Tuin, 2016), covering a range of classes and generations which are currently used in both human and veterinary medicine. Antibiotics were grouped as per previous research into 4 panels: Veterinary, Extended Spectrum Beta Lactamase A, Extended Spectrum Beta Lactamase B and Clinical panel (Table 2) (Tuin., 2016).

Table 2: Antimicrobials identified by the previous student used in AST categorised by panel.

	Panel			
	Vet	**ESBL A**	**ESBL B**	**Clinical**
Antimicrobial (µg/disc)	25 Amoxicillin	5 Cefotaxime	10 Ceftazidime	5 Ciprofloxacin
	10 Ampicillin	10/20 Amoxicillin/Clavulanic	30 Cefepime	10 Gentamicin
	30 Oxytetracycline	30 Piperacillin	10 Ertapenem	15 Tigecycline
	5 Marbofloxacin	30/6 Piperacillin/Tazobactam	10 Meropenem	
		30 Cefuroxime	10 Imipenem	
		30 Cefoxitin		
		30 Cefotaxime		

Antibiotic used in susceptibility testing

Overnight cultures were incubated after which colonies were aseptically transferred into 5 ml aliquots of sterile Ringers (Sigma Aldrich UK) solution, forming a bacterial suspension to a 0.5 McFarland standard determined by visual turbidity. Sterile cotton swabs were used to transfer suspension onto the MHA plates (Sigma Aldrich UK) as per EUCAST methodology (Matuschek *et al*., 2014) and left for a maximum of 5 minutes for inoculum to dry. All antibiotic discs apart from marbofloxacin (Mast diagnostics UK) were purchased from the same supplier (OXOID Ltd UK), were aseptically placed onto the inoculated MHA using an antibiotic disc dispenser (OXOID Ltd) equidistant from each other with a maximum 3 per plate to prevent ZOI overlap. This was done within 15 minutes of inoculation followed by plate inversion. Plates were then incubated for 24 hours at 37°C and read the following day with the diameter of no bacterial growth used to determine the ZOI using a digital calliper (J-bonest GD00017).

Sodium Pyruvate

Sodium pyruvate (NaPyr) solution was prepared to a final concentration of 0.01% w/v solution using sterilised deionised water and filter sterilised through a 0.22μm filter (Sigma Aldrich UK). NaPyr solution was transferred aseptically into sterile molten MHA which had been cooled to handheld temperature prior to pouring and decanted to 4mm in 90mm Petri dishes (SLS Laboratories UK, Fisher Scientific UK). MHA plates were allowed to set, and surface moisture removed prior to use as above. AST was carried out on overnight cultures using the method above on MHA, MHCA and MHA+NaPyr for comparison and incubated for 24 hours at 37°C. ZOI (mm) was measured the following day using the digital calliper.

2.5 Re-establishing antimicrobial resistance

Of the selected *P. aeruginosa* and *E. coli* isolates, several changes in antimicrobial resistance profiles were seen from original research, in particular increased susceptibility where the isolate previously tested resistant. Overnight cultures were prepared from -80°C stocks onto NA and passaged twice from which a bacterial suspension was prepared equivalent to a 0.5 McFarland standard in sterile Ringers solution. MHA was prepared as above and an antimicrobial disc was placed in the centre of the agar plate and inoculated with bacterial suspension, using a sterile cotton swab, outwards from the centre of the antimicrobial disc. Plates were inverted and incubated at 37°C for 24 hours. The process was then repeated with cells taken from the edge of the Zone of Inhibition and suspended in Ringers solution to a 0.5 McFarland standard and plates were incubated for a further 24 hours.

AST was repeated using the method above and incubated for 24 hours at 37°C. ZOI (mm) was then read the following day using a digital calliper and compared to

EUCAST v9 (2019) and previous ZOI obtained to confirm antimicrobial resistance profile.

2.6 Establishing a point of exponential growth and efflux inhibitor assay

Overnight cultures were prepared on NA and incubated for 24 hours at 37°C from frozen stock. MHB (OXOID Ltd) and MHCB (OXOID Ltd) was prepared as per manufacturing guidelines and decanted into 5ml aliquots in universal containers and 50 ml aliquots in 250 ml conical flasks followed by sterilisation at 121°C at 15 psi for 15 minutes. Single colonies from overnight culture were transferred to the sterile broths and incubated for 24 hours at 37°C. From overnight broth cultures, 50 µl was transferred into 50 ml sterile broth in 250 ml conical flasks and placed into a Unimax 1000,1010 shaking incubator (Heidolph Ltd UK) at 37°C and 250 rpm. A 2ml aliquot was transferred aseptically at 30-minute intervals with 1ml of the aliquot transferred to plastic cuvettes (SLS Laboratories UK) to measure Optical Density at 600_{nm} using a Helios Epsilon spectronic spectrophotometer which was blanked using broth of the same composition which was sterile (ThermoFisher Scientific, UK). The remaining 1ml aliquot was suspended into sterile Ringers solution for serial dilution and colony counting. Inoculated Ringers solution was serial diluted and 100 µl transferred to the respective MHA and MHCA and spread using sterile cotton swabs and incubated at 37°C for 24 hours. Colonies were counted the following day from dilution of 10^{-2} to 10^{-7}. Growth curve was only taken to an absorbance of 1 following indication of an absorbance of around 0.6 for *P. aeruginosa* (Yang *et al*., 2018) and 0.4 to 0.6 for *E. coli* (Kobayashi *et al.,* 2006). This was also supplemented with the colony counting as per the EUCAST MIC (EUCAST: EUCAST broth microdilution reading guide updated, 2021) and CLSI methodology (Cockerill, 2012).

PaβN (Sigma Aldrich UK) stock solution was prepared aseptically using SDW and stored in 2.5ml aliquots at -20°C. The final concentration of PaβN used was 10µg/ml. Overnight cultures were prepared on NA from frozen stocks and passaged twice with incubation for 24 hours at 37°C between each passage. Four conical flasks containing 50ml sterile broth were set up and brought up to room temperature containing one of the following: MHB; MHB+PaβN; MHCB; MHCB+PaβN. Single colonies from purity plates were suspended into 10mls of sterile MHB and MHCB with and without 0.1 mls of a 1 mg/ml solution of PaβN, (PaβN; Sigma Aldrich UK) and incubated for 24 hours at 37°C. From the overnight inoculated broths 50µl of inoculum was transferred to 5 0mls of the respective sterile broth in 250 ml conical flasks with the addition of 0.5 mls of a 1 mg/ml PaβN solution. An aliquot of 2 mls was taken from the inoculated broth within the conical flask at T_0 and serially diluted for colony counting. The inoculated conical flasks were incubated on the shaking incubator at 250 rpm for 2.5 hours at 37°C (time determined from growth curve). A 1 ml aliquot was suspended into 10 mls of sterile Ringers solution and colony counted onto nutrient agar to confirm a point of exponential growth following incubation. 100µl of inoculum was transferred from the inoculated conical flask to respective agar plates (MHA, MHA+ PaβN, MHCA and MHCA+ PaβN) and spread using a sterile cotton swab giving a final $1x10^5$ CFU/ml as per EUCAST and CLSI guidelines (Hasselmann, 2003, EUCAST: EUCAST broth microdilution reading guide updated, 2021, Cockerill, 2012).

A MIC E-TEST (Biomerieux) was performed with a representative antibiotic of each class (Table 3) and aseptically placed onto the centre of the inoculated agar before plates were inverted and incubated for 18 hours at 37°C. The intersection of growth

on the MIC E-TEST was read to determine Minimum inhibitory concentration in µg/ml (MIC).

Table 3: MIC E-TEST representative of each class of antimicrobial used for efflux pump inhibitor with concentration ranges for each strip.

Antimicrobial E-TEST	MIC Concentration range (µg/mL)
FX - cefoxitin	0.016 - 256
TZ - ceftazidime	0.016 - 256
PM - cefepime	0.016 - 256
CI - ciprofloxacin	0.002 -32
IP - imipenem	0.002 - 32
AC - amoxicillin	0.016 - 256
XL – amoxicillin / clavulanic acid	0.016 - 256
TC – tetracycline	0.016 - 256

3. Results

The total number of isolates initially tested in this project were randomly selected and totalled 113, with 82 *E. coli* and 31 *P. aeruginosa* (full initial AST data available in appendix 2). This can then be split further between bovine gut (BG) and environmental isolates, 63 and 19 for *E. coli* and 14 and 17 for *P. aeruginosa,* respectively. Of the purified and presumptive identified isolates by the previous KU student, approximately one third of each species was selected for this research project, which were distributed between the three farms and isolate origin of location (Figure 1) for conformational AST. Each isolate was retested to determine antibiotic susceptibility via the EUCAST methodology, with ZOI compared against EUCAST (V9.1) breakpoints.

3.1 Antibiotic susceptibility testing

Following isolate growth from frozen stock, the resistance profiles were checked and compared to NCTC strains of both *P. aeruginosa* (NCTC 13359) and *E. coli* (NCTC 11954). These were compared to EUCAST breakpoints v9.1 (01/2019) to determine isolate susceptibility profiles. During the testing of these isolates for initial antibiotic susceptibility Marbofloxacin discs were unavailable at the time of testing however isolates were tested in triplicate against this antibiotic later once it became available. Antibiotics utilised in this project (Table 4) segregated by antibiotic class.

Table 4: Antibiotics identified and utilised in AST testing for antibiotic susceptibility confirmation which are grouped by class. β-lactamase inhibitors combination with antibiotics are included within β-lactam class.

Antimicrobial Class					
β-lactam	Tetracycline	Carbapenem	Cephalosporin	Aminoglycoside	Fluoroquinolones
Amoxicillin	Oxytetracycline	Ertapenem	Cefuroxime (2nd)	Gentamicin	Ciprofloxacin
Ampicillin	Tigecycline	Imipenem	Cefotaxime 5µg (3rd)		Marbofloxacin
Cefoxitin		Meropenem	Cefotaxime 30µg (3rd)		
Piperacillin			Ceftazidime (3rd)		
Piperacillin-Tazobactam			Cefepime (4th)		
Amoxicillin-Clavulanic acid					

With the first antibiotic discovered and mass produced in the 1940s and 50s, β-lactam and tetracycline resistance were among the first to be observed in clinical settings soon after their discovery. Resistance to both β-lactams and tetracycline was seen in both the BG and ENV isolates holding high percentages of resistance towards oxytetracycline.

Table 5: Total number of *E. coli* isolates tested from Bovine Gut and Environmental locations resistant to each antibiotic in addition to *E. coli* NCTC 11954 to represent a typed strain.

Antibiotic panel	Antimicrobial Disc	Number of *E. coli* isolates showing antibiotic resistance (%)		
		Bovine Gut (N=63)[1]	Environmental (N=19) [1]	NCTC 11954 (N=1) [1]
Veterinary	Amoxicillin	4 (6.3%)	8 (42.1%)	1(100%)
	Ampicillin	5 (7.9%)	8 (42.1%)	1(100%)
	Oxytetracycline	15 (23.8%)	12 (63.2%)	0%
	Marbofloxacin	N/A	N/A	0%
ESBL A	Cefotaxime	0	1 (5.3%)	0%
	Cefotaxime	0	0	0%
	Amoxicillin +Clavulanic acid	2 (3.2%)	4 (21.1%)	1(100%)
	Piperacillin	2 (3.2%)	3 (15.8%)	1(100%)
	Piperacillin +Tazobactam	0	1 (5.3%)	0%
	Cefuroxime	1 (1.6%)	3 (15.8%)	0%
	Cefoxitin	2 (3.2%)	4 (21.1%)	0%
ESBL B	Ceftazidime	2 (3.2%)	0	0%
	Cefepime	3 (4.8%)	1 (5.3%)	0%
	Ertapenem	1 (1.6%)	0	0%
	Meropenem	0	0	0%
	Imipenem	0	0	0%
Clinical	Ciprofloxacin	0	0	0%
	Gentamicin	1 (1.6%)	0	0%
	Tigecycline	1 (1.6%)	1 (5.3%)	0%

1N= number of isolates undergone full AST

Antibiotic resistance in environmental isolates of *E. coli* was seen with 40% of environmental isolates tested showing resistance towards amoxicillin and ampicillin within the veterinary panel, however resistance towards the ESBL A panel was seen with at least one isolate from BV and ENV and amoxicillin/clavulanic acid showing greater resistance in ENV isolates (Table 5). A lack of fluoroquinolone resistance in the *E. coli* isolates tested including the NCTC strain (Table 5, Table 6) is favourable as it is one of the last classes of antibiotics to be developed.

Interestingly BG isolates showed no resistance towards 3rd generation cephalosporins (ceftazidime and cefotaxime) however some decrease in susceptibility was observed in the 4th generation (cefepime). The environmental isolates showed no carbapenem and aminoglycoside resistance however some

resistance was observed to cefotaxime at 5 µg however this was overcome when increased to 30 µg with all isolates seen to be susceptible (Appendix 2). The structure of β-lactams and cephalosporins both contributing to AMR in both clinical and veterinary settings with binding to β-lactamases occurring from points close to the discovery of the antibiotics within the classes and resistance mediated by the acquisition of enzymes (Bush and Bradford, 2016).

Table 6: Percentages of *E. coli* isolates resistant to at least one antibiotic in the class.

	E. coli	
	Bovine Gut (N=63)[1]	Environmental (N=19)[1]
Isolates showing β-lactam resistance	8 (12.7%)	8 (42.1%)
Isolates showing tetracycline resistance	16 (25.4%)	12 (63.2%)
Isolates showing carbapenem resistance	1 (1.6%)	0
Isolates showing cephalosporin (generation 2-4) resistance	5 (7.9%)	4 (21.1%)
Isolates showing fluoroquinolone resistance	0	0
Isolates showing aminoglycoside resistance	16 (25.4%)	3 (15.8%)

1 N= is based on the representative number of isolates tested which were available at the time.

From the representative sample of *E. coli* isolates tested against the 19 antibiotics (Table 4), >50% of ENV isolates and >25% of BG isolates tested held tetracycline resistance in addition to >10% of isolates holding β-lactam resistance.

P. aeruginosa is an opportunistic pathogen for both humans and animals with clinical and veterinary strains of this species known to hold resistance towards a variety of classes of antibiotics including tetracyclines, β-lactams and aminoglycosides (Lister *et al.*, 2009, Hirsch and Tam, 2010).

Table 7: Total number of *P. aeruginosa* isolates tested from Bovine Gut and Environmental locations resistant to each antibiotic in addition to *P. aeruginosa* NCTC 13359 to represent a typed strain.

Antimicrobial panel	Antimicrobial Disc	Antibiotic resistance in *P. aeruginosa* isolates		
		Bovine Gut (N=14)[1]	Environmental (N=17)[1]	NCTC 13359 (N=1)[1]
Veterinary	Amoxicillin	0	8 (47.1%)	1 (100%)
	Ampicillin	0	7 (41.2%)	1 (100%)
	Oxytetracycline	11 (78.6%)	7 (41.2%)	0
	Marbofloxacin	0	0	0
ESBL A	Cefotaxime	1 (7.1%)	2 (11.8%)	1 (100%)
	Cefotaxime	0	1 (5.9%)	1 (100%)
	Amoxicillin +Clavulanic acid	0	1 (5.9%)	1 (100%)
	Piperacillin	0	0	0
	Piperacillin +Tazobactam	0	1 (5.9%)	0
	Cefuroxime	1 (7.1%)	3 (17.6%)	1 (100%)
	Cefoxitin	3 (21.4%)	3 (17.6%)	1 (100%)
ESBL B	Ceftazidime	2 (14.3%)	0	0
	Cefepime	0	2 (11.8%)	0
	Ertapenem	2 (14.3%)	4 (23.5%)	1 (100%)
	Meropenem	0	0	0
	Imipenem	0	1 (5.9%)	0
Clinical	Ciprofloxacin	1 (7.1%)	0	0
	Gentamicin	0	0	0
	Tigecycline	0	0	0

2N= number of isolates undergone full AST

Initial antibiograms indicate that >40% of *P. aeruginosa* isolates held resistance to oxytetracycline within the veterinary panel (Table 7) with isolates from both BG and ENV locations. No other resistance was observed in the veterinary panel of BG isolates however ENV isolates of *P. aeruginosa* showed resistance towards most of the veterinary panel of antibiotics excluding marbofloxacin which was tested on all isolates in triplicate once it became available. The NCTC strain showed resistance to β-lactams in both veterinary and ESBL A panels with piperacillin and piperacillin-tazobactam the only β-lactams of which susceptibility was observed, although the BG isolates only showed resistance to cefoxitin and showed susceptibility to the other β-lactams. Carbapenem resistance was seen in both BG and ENV isolates

initially in addition to the NCTC strain with ertapenem in the ESBL B panel (Table 7 and 8). *P. aeruginosa* showed no fluoroquinolone resistance in both the environmental and NCTC isolates and complete antibiotic susceptibility towards aminoglycoside.

Table 8: Percentages of *P. aeruginosa* isolates resistant to at least one antibiotic in the class.

	P. aeruginosa	
	Bovine Gut (N=14) [1]	Environmental (N=17) [1]
Isolates showing β-lactam resistance	3 (21.4%)	9 (52.9%)
Isolates showing tetracycline resistance	11 (78.6%)	7 (41.2%)
Isolates showing carbapenem resistance	2 (14.3%)	4 (23.5%)
Isolates showing cephalosporin (generation 2-4) resistance	3 (21.4%)	6 (35.3%)
Isolates showing fluoroquinolone resistance	1 (7.1%)	0
Isolates showing aminoglycoside resistance	0	0

1. N= is based on the representative number of isolates tested which were available at the time.

Susceptibility testing on isolate storage stock

Changes in isolate susceptibility towards the panels of antimicrobials was observed between different experiments and the creation of new bacterial stocks, of which increased susceptibility (loss of resistance) was the most common change observed more specifically towards aminoglycosides in both *E. coli* and *P. aeruginosa*.

From the initial 113 bacterial isolate stocks, 10 isolates were selected of each species with equal number from BG and ENV locations with changes in the susceptibility identified towards at least ONE antimicrobial following the confirmational AST (Table 9). With *P. aeruginosa* increases in susceptibility was observed in both ENV and BG isolates to all the antimicrobials being tested apart from oxytetracycline. *E. coli* showed a mixture of increases and decreases of susceptibility as each new bacterial stock was created with some isolates showing

increases in β-lactam resistance with amoxicillin/clavulanic acid and cefoxitin however, increased susceptibility was seen with ampicillin, and decreases in cephalosporin resistance with increased susceptibility towards cefepime and cefuroxime. An increase in carbapenem susceptibility within the BG isolates observed with increased susceptibility in all of the *E. coli* isolates stocks created.

Table 9: Number of *P. aeruginosa* and *E. coli* isolates resistant to each antimicrobial selected for continuation within the study.

Antimicrobial panel	Antimicrobial	*E. coli*[1,2]		*P. aeruginosa*[1,2]	
		BG	ENV	BG	ENV
Veterinary	Amoxicillin	0	2	1	1
	Ampicillin	1	2	0	1
	Oxytetracycline	3	2	2	2
	Marbofloxacin	0	0	0	0
ESBLA	Cefotaxime	2	1	0	0
	Cefotaxime	0	0	0	0
	Amoxicillin +Clavulanic acid	3	2	1	1
	Piperacillin	0	0	0	0
	Piperacillin +Tazobactam	0	0	0	0
	Cefuroxime	0	1	1	1
	Cefoxitin	4	1	0	1
ESBLB	Ceftazidime	0	2	0	0
	Cefepime	0	1	0	0
	Ertapenem	0	0	0	0
	Meropenem	0	0	0	0
	Imipenem	0	0	0	0
Clinical	Ciprofloxacin	0	0	0	0
	Gentamicin	0	1	0	0
	Tigecycline	0	0	1	0

1 BG (n=5) and ENV (n=5)

2 Number of isolates resistant to each antimicrobial following confirmational AST

The selected *P. aeruginosa* isolates showed an overall decrease in susceptibility towards at least one antimicrobial within the antimicrobial panel with all isolates tested showing increase in susceptibility towards the ESBL B and Clinical panel in-particular (Table 9). A decrease in β-lactam susceptibility was observed within the environmental isolates with contrast to an increase in β-lactam susceptibility with the BG isolates.

Table 10: Number of isolates resistant to each class of antimicrobial at the final bacterial stocks created

	E. coli [1,2]		*P. aeruginosa* [1,2]	
	Bovine Gut	Environmental	Bovine Gut	Environmental
Number of isolates showing β-lactam resistance	2	2	4	2
Number of isolates showing tetracycline resistance	2	2	3	2
Number of isolates showing carbapenem resistance	0	0	1	0
Number of isolates showing cephalosporin resistance	0	1	2	3
Number of isolates showing fluoroquinolone resistance	0	0	0	0
Number of isolates showing aminoglycoside resistance	0	0	0	0

1 N=5 for BG and Environmental isolates. N= Total number of isolates of each species carried forward for progression through investigation

2 Number of isolates resistant to each class of antibiotic following preparation of new bacterial stock and full AST

Through the creation of the initial 119 bacterial stock and the final bacterial stocks of the selected isolates to continue through the project (20), changes in susceptibility to each antimicrobial in of all the *P. aeruginosa* and *E. coli* isolates was observed with complete loss aminoglycoside resistance and any fluoroquinolone resistance seen (Table 9 and 10). Fluctuation in the susceptibility was seen between the creation of each bacterial stock with some isolates gaining and losing antibiotic susceptibility during this project, thus the need to restore bacterial resistance profiles was required with an adaptation of the method used by Dore *et al.* (1999) using antimicrobial concentrations between MIC and MBC to identify colonies showing resistance leading to the development of the antimicrobial exposure assay.

3.2 Re-establishing Antibiotic resistance

Changes in antibiotic susceptibility was observed in 6 of the *E. coli* bacterial stocks and 8 of the *P. aeruginosa* bacterial stocks. Of the 10 *P. aeruginosa* and 10 *E. coli,* isolates selected to move forward with this project (5 BG and 5 ENV), changes in susceptibility towards at least one antimicrobial was identified and required the assay mentioned above with subsequent AST against the full panel of antibiotics (Table 4) to re-establish and confirm antimicrobial susceptibility profiles. Four BG and two of ENV *E. coli* isolates in addition to three BG and five ENV *P. aeruginosa* isolates required this assay to restore previously identified antibiotic resistance.

From the selected isolates, the majority had increased susceptibility towards at least 1 antibiotic (Table 11). The antibiotic exposure assay would allow for colonies to be cultured from the edge of the ZOI with the aim of selecting colonies with decreased susceptibility towards the antibiotic. The antibiotic exposure assay was carried out twice for each isolate to recover the original resistance pattern.

Table 11: The number of *E. coli* and *P. aeruginosa* which decreased antimicrobial susceptibility following the antibiotic exposure assay and full AST

Antimicrobial panel	Antimicrobial	*E. coli*[2]		*P. aeruginosa*[2]	
		BG (n=4)[1]	ENV (n=2)[1]	BG (n=3)[1]	ENV (n=5)[1]
Veterinary	Amoxicillin	0	1	0	2
	Ampicillin	0	1	1	3
	Oxytetracycline	1	2	2	3
	Marbofloxacin	0	0	0	0
ESBLA	Cefotaxime	0	0	1	0
	Cefotaxime	0	0	0	0
	Amoxicillin +Clavulanic acid	0	0	2	2
	Piperacillin	0	0	0	0
	Piperacillin +Tazobactam	0	0	0	0
	Cefuroxime	0	0	0	1
	Cefoxitin	0	0	2	2
ESBLB	Ceftazidime	0	0	1	2
	Cefepime	0	0	1	1
	Ertapenem	0	0	1	0
	Meropenem	0	0	0	0
	Imipenem	0	0	0	1
Clinical	Ciprofloxacin	0	0	1	0
	Gentamicin	0	0	0	0
	Tigecycline	1	0	0	0

1. N equates to the number of isolates which had changes in resistance towards at least one antimicrobial compared to initial AST.

2 Full data following antimicrobial exposure assay available in appendix 4 with full AST data against all 4 panels of antimicrobials

Note number equates to number of isolates which were resistant to the antimicrobial after the full AST. Full AST data in appendix 4

Post assay AST showed decreased susceptibility towards some antimicrobials in particular β-lactams, with the antimicrobial resistance recovered in *P. aeruginosa* isolates from both locations. Some carbapenem resistance was observed in isolates from both locations, however no fluoroquinolone and aminoglycoside resistance was observed in any of the *P. aeruginosa* or *E. coli* isolates post assay which was identified. The carbapenem resistance initially detected by the previous KU student and identified initially in the AST confirmation in 2 of the BG *P. aeruginosa* isolates as well as 2 ENV and 1 BG *E. coli isolate* was no longer present in the final bacterial stock. (appendix 4).

The antibiotic exposure assay data obtained from *E. coli* isolates showed it was not as effective as it was with *P. aeruginosa* isolates, with only 2 antibiotics having decreased susceptibility from BG isolates and 3 antimicrobials with decreased susceptibility within environmental isolates. However, post antimicrobial exposure assay AST indicates that both of the environmental *E. coli* isolates that were undertaken within the antimicrobial exposure assay had gained resistance to oxytetracycline which were previously susceptible. This was also seen in an ENV *P. aeruginosa* isolates which had been previously tested susceptible (Table 11).

Some isolates gained antibiotic resistance which had previously been lost where some isolates lost resistance which was not recovered following double exposure of the assays and AST testing. No carbapenem resistance was seen after the antibiotic exposure assay and AST testing which followed from isolates which initially held resistance to ertapenem, meropenem or imipenem. The *E. coli* BG isolates were seen to become more susceptible to β-lactam and tetracycline antibiotics within the veterinary panel (Table 11) however all the ENV *E. coli* isolates showed resistance to tetracycline post antibiotic exposure assay AST (Table 11).

P. aeruginosa showed increases in resistance in both ENV and BG isolates post antibiotic exposure assay in addition to EBSL B antibiotic panel which constitutes mainly carbapenem antibiotics (Table 4, Table 11). An increase β-lactam resistance was also seen in the *P. aeruginosa* isolate towards ESBL A and veterinary panels however a loss of resistance was seen in BG isolates towards amoxicillin post antibiotic exposure assay AST. Post antibiotic exposure assay AST 10 *P. aeruginosa* and 10 *E. coli* isolates had restored some of the resistance profiles which were

identified at the beginning of the project and had then increased in susceptibility in addition to increase in resistance towards antibiotic in some of the isolates which were previously shown to be susceptible. Post assay AST data available in appendix 4.

Sodium pyruvate and cation adjusted media

Cation adjusted MH media was purchased commercially (OXOID,UK) and prepared within the laboratory as per manufacturing guidelines. Cation adjusted Mueller-Hinton is media which has added Ca^{2+} and Mg^{2+}, so that it falls into the recommended range by EUCAST and CLSI (Appendix 1) in dehydrated form prior to reconstitution.

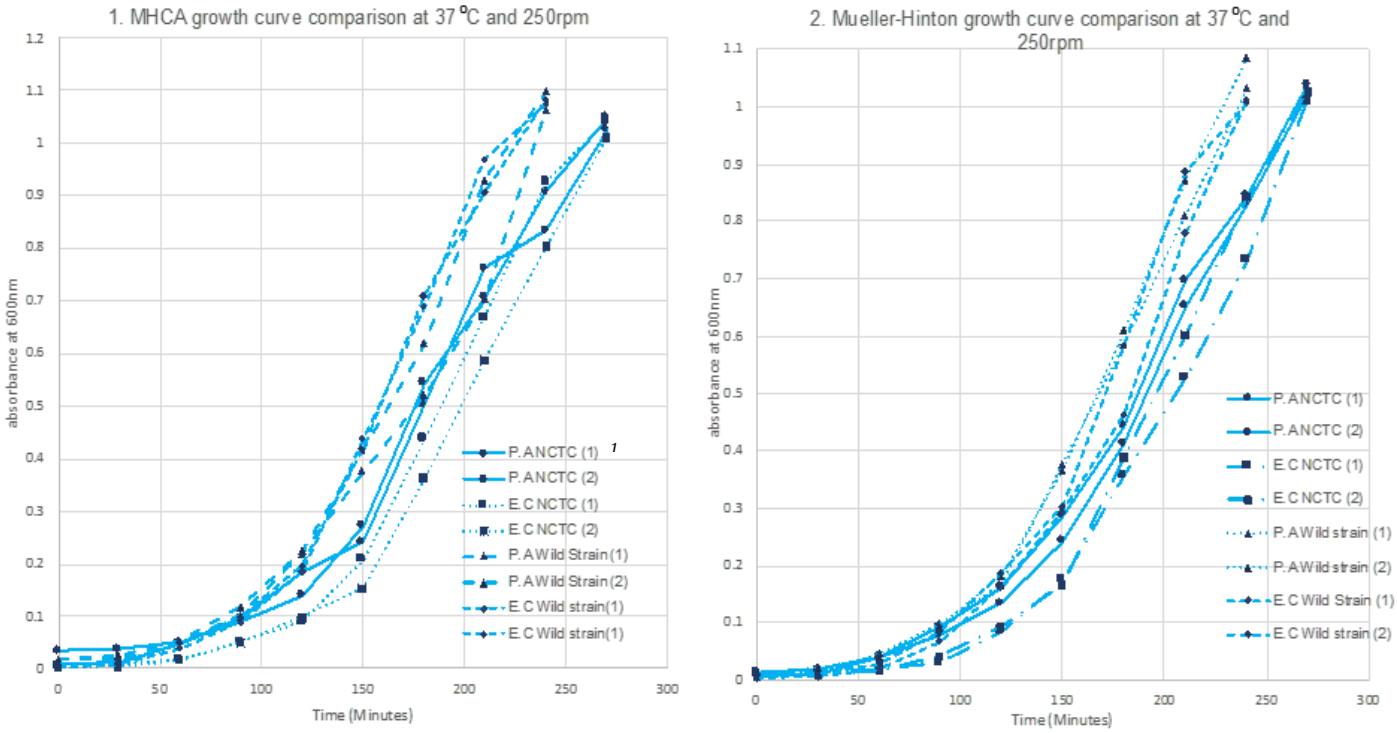
NaPyr was used as an alternative carbon energy source in Mueller-Hinton agar used for AST. NaPyr has traditionally been used to recover cells which had undergone thermal stress (Czechowicz *et al*., 1996). Su *et al* (2018) demonstrated that the pyruvate cycle can be targeted to increase the efficacy of aminoglycosides when tackling resistant *E. coli* pathogens. Here the testing of NaPyr was used to supplement the pyruvate cycle in an attempt to investigate changes in susceptibility of *E. coli* and *P. aeruginosa* isolates towards four panels of antimicrobials.

The use of cation adjusted Mueller-Hinton agar acts as a supplement of divalent cations Mg^{2+} and Ca^{2+} are used in outer membrane stability interacting with the accumulation of negative charge provided by the discharge of positive charge from surrounding lipopolysaccharides (LPS). The divalent cations bind to the anionic phosphate and carboxylic groups LPS to maintain integrity and help to contribute to AMR by preventing hydrophobic antimicrobial agents from entering Gram-negative bacterial cells (Clifton *et al*., 2014).

Forty isolates were randomly selected initially from the available isolates identified by the previous KU student, with selection between *P. aeruginosa* and *E. coli*. Isolates were also selected with a spread between resistant and susceptible profiles in addition to ENV or BG origin. AST was done with NaPyr supplement on normal MHA and MHCA to compare isolate susceptibility towards the antimicrobial panel and as to whether the use of alternate carbon source for energy would affect the susceptibility of the antimicrobials measured as ZOI. The addition of NaPyr at a 0.01% w/v showed decrease in antimicrobial susceptibility towards gentamicin in 2 of the *E. coli* agricultural isolates shown in appendix 3, thus further testing with a higher concentration is required. Pyruvate susceptibility testing in appendix 3.

3.3 Establishing a point of exponential growth and efflux inhibitor assay

Growth curves were performed on BG and ENV isolates of *P. aeruginosa* and *E. coli* in addition to their NCTC strains to identify a point of exponential growth to perform the EPI-MIC testing. Growth curves were performed in both Mueller-Hinton and Mueller-Hinton cation adjusted media to observe any variation in time to reach exponential phase growth at 37°C at 250rpm. Growth curve measurements by optical density at 600_{nm} was supplemented with colony counting to find ~1-2 $x10^6$ CFU/ml (appendix 5). A time of 2.5 hours was identified during the growth curve giving an OD between 0.2 to 0.5 (Figure 7) giving a CFU/ml of roughly 10^6 through colony counting supplementation.



1 Growth curve of NCTC and veterinary isolates of P. aeruginosa and E. coli. P.A = P. aeruginosa, E.C= E. coli.

Figure 7: Growth curves on MHCA and MHA to identify exponential growth phase growth. Curves only taking to an absorbance of 1 as target absorbance between o.4-0.6 was identified from literature for both species, as such curves were not taken to stationary phase. Colony counting supplementation through the growth curve to achieve a 10^6 CFU/mL as per EUCAST methodology. Colony count data supplemented (appendix 4).

A final concentration of 10ug/mL of PAβN was utilised as it had been observed to cause a decrease by at least 1-fold within the MIC of various antimicrobials and compounds as mentioned above. A mixture of both environmental and BG isolates were randomly selected and tested against 1 antimicrobial randomly selected from each class of antimicrobial as representative of the class of antimicrobials (Table 4). Variation was observed with the MICs between each individual isolate. The MIC was compared to breakpoints obtained from EUCAST v9.1 (2019) and CLSI Vet08 (2019). Of the *E. coli* isolates tested, one of the isolates originating from BG and environment showed complete resistance to tetracycline on both MHA and MHCA with and without the presence of PAβN. A *P. aeruginosa* originating from BG

showing no susceptibility apart from one isolate which showed susceptibility on MHCA however again without the EPI, this may be down to the presence of cations within the media, but further testing is needed. Isolate 5 of *P. aeruginosa* of BG origins showed a decrease in MIC, 1 µg/mL to 0.75 µg/mL from MHA to MHCA for ceftazidime and a decrease In the MIC from 1 µg/mL to 0.5 µg/mL with MHCA+PAβN. *P. aeruginosa* isolate 6 showed a greater decrease in MIC with the presence of PAβN without the cations than with the presence of cations and PAβN (Figure 8.) A MIC of 4 µg/mL was seen with MHCA+ PAβN and an MIC of 0.032 µg/mL was seen on MHA+ PAβN, which could indicate that presence of cations could affect the susceptibility of cefepime on this isolate.

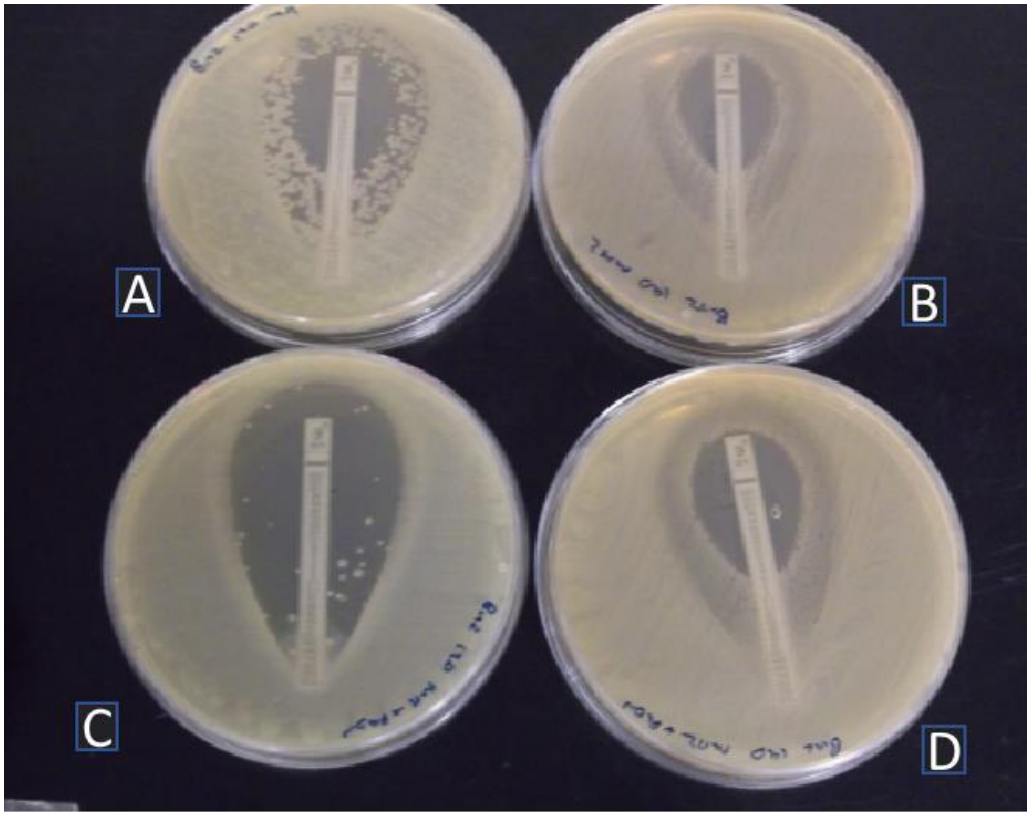


Figure 8: E-TEST MIC result for isolate 6 *P. aeruginosa* for amoxicillin-clavulanic acid. A) MH agar, B) MHCA agar, C) MHA agar + PAβN, D) MHCA agar + PAβN. Concentration of 10 µg/mL utilised of PAβN. ZOI shown post incubation and read at intersection of E-TEST.

E. coli isolate 2 from environmental origins showed a decrease in MIC of cefepime from 0.125 µg/mL to 0.064 µg/mL on MHA and a decrease on MHCA in addition a two-fold decrease from 0.125 µg/mL to 0.047 µg/mL with PAβN was observed compared to normal MHA. Both *E. coli* and *P. aeruginosa* showed decreases in their MIC using MHCA in addition to isolates from BG of both species showing around a twofold decrease in MIC with MHCA and PAβN (Table 12).

Overall, both organisms showed no decrease in MIC with the presence of cations or PAβN at the concentration utilised towards β-lactam antibiotics (amoxicillin, cefoxitin) including those with β-lactamase inhibitors (Amoxicillin- clavulanic acid). Further testing using PAβN at a range of concentrations and varied concentrations of cations is required. Full MIC data is provided appendix 5.

Table 12: E-TEST MIC for veterinary isolates shown for antimicrobials with decrease in MIC (µg/ml). Full MIC data available in the appendix 5.

Isolate No.	Species[1]	*Sample*[2] origin	Imipenem				Ceftazidime				Ciprofloxacin				Cefepime			
			MH	MH + PAβN[3]	MH2	MH2 + PAβN[3]	MH	MH + PAβN[3]	MH2	MH2 + PAβN[3]	MH	MH + PAβN[3]	MH2	MH2 + PAβN[3]	MH	MH + PAβN[3]	MH2	MH2 + PAβN[3]
1	E.C	Env	0.094	0.19	0.094	0.38	<0.016	0.125	0.023	1	0.012	0.003	0.004	0.064	0.016	0.094	0.016	0.094
2	E.C	Env	0.75	0.38	0.38	1	0.064	0.047	0.032	0.25	0.064	0.064	0.047	0.094	0.125	0.064	0.047	0.064
3	E.C	BG	NZ	2	1	0.75	0.5	0	0.19	1.5	0.016	0.016	0.016	0.016	0.064	0.094	0.023	1
4	P. A	Env	0.125	0.25	0.094	0.19	<0.016	<0.016	<0.016	<0.016	<0.002	<0.002	<0.002	<0.002	0.023	0.047	0.023	0.064
5	P. A	BG	NZ	2	0.5	1.5	1	1	0.75	0.5	0.032	0.023	0.016	0.016	0.047	0.094	0.064	0.125
6	P. A	BG	1.5	1	0.19	4	32	32	35	48	0.25	0.01	0.5	0.25	6	0.032	6	4

1. E.C – *E. coli*, P.A – *P. aeruginosa*
2. Env – Various environmental locations around the dairy farms, BG – Samples obtained from Bovine Guts of dairy producing cattle.
3. EPI used PAβN at a final concentration of 10 µg/ml
NB: Media abbreviations: MH - Mueller-Hinton agar, MH+PAβN – Mueller-Hinton agar with EPI, MH2 - Mueller-Hinton Cation adjusted agar, MH2+PAβN – Muller-Hinton cation adjusted agar with EPI.
Note. all values are in µg/ml, NZ – No readable zone identified following incubation

4. Discussion

Both *E. coli* and *P. aeruginosa* have shown cross-species pathogenicity causing severe infections in humans and within the veterinary industry. The presence of various *E. coli* strains can be seen to be symbiotic in both human and bovine gut flora, however *E. coli* is able to cause cross species infections (Arimizu *et al*., 2019). Both *P. aeruginosa* and *E. coli* have developed MDR from incorrect use and prolonged exposure of antibiotics from treatments in both human and veterinary medicine.

In this study, initial antibiotic susceptibility of a representative sample of *P. aeruginosa* and *E. coli* isolates equally distributed between BG and ENV origins was selected from the library of isolates available, in addition to a NCTC typed strain against 4 panels containing a total of 19 antibiotics covering 6 classes. AST was done performed using MHA agar to confirm previous susceptibility profiles in addition to and MHCA to identify any changes in the ZOI occurred when running AST on cation supplemented media. Once the antibiotic susceptibility profiles of the isolates had been confirmed, growth curves supplemented with colony counting was performed to reach lag phase with and without the presence of cations and the addition of EPI. E-TEST MIC was then performed with and without cations and EPI to determine whether susceptibility of the isolates changed towards a class representative antibiotic.

Antimicrobial susceptibility testing

All breakpoints were compared to EUCAST rather than CLSI for reasons of continuity due to variations between ZOI recommendations and for consistency with the previous student's work and study of the isolates.

Initial susceptibility testing of both species NCTC typed strains and environmental isolates showed resistance to β-lactam antibiotics. Increase in antibiotic susceptibility was observed in both species through the creation of new bacterial stocks. This could be due to various reasons; sublethal injury, colonies selected may have been susceptible, damage to bacterial cells during transfer, over passage of isolates or possible contamination of created stocks and deletion of transposons or plasmid (Wesche *et al*., 2009; Mackey, 1983).

Loss of antibiotic resistance

Isolates which had been identified to have lost resistance towards at least one antibiotic were then exposed to an antibiotic exposure assay in an attempt to restore antibiotic susceptibility profiles initially identified. This was checked with AST for confirmation and comparison against previous susceptibility profile of the isolate being tested. Of which some restoration of previous susceptibility profiles was achieved, in addition to new antibiotic resistance had appeared in some of the isolates towards different antimicrobials from a phenotypic point. This was seen to be both positive and negative with *E. coli* showing increases in resistance in both environmental and BG isolates in addition to *P. aeruginosa* environmental isolates however loss of resistance was seen in BG isolates, this could be due to the loss of resistance genes (Kim et al., 2021) or changes in active efflux pumps and presence of porins as seen by Fernandez and Hancock (2012). This could also be due to mixed susceptible colonies within the bacterial stocks as well as possible errors in the initial reporting of susceptibility profiles or mixed colonies when preparing new bacterial stock.

With all organisms having some water content, the storage of cells around 0°C allows for the formation of ice crystals within the cells causing changes in nucleation and metabolic pathways (El-kest and Marth, 1992) thus allowing rise to cellular injury and thermal shock. This can lead to accumulation of cell solutes which causes dissociation of lipoproteins during the rapid freezing (El-kest and Marth, 1992). The addition of cryoprotectant with cold storage minimizes the dehydration of cells, reducing damage to cell membranes preventing osmotic shock (Meryman, 1971) and exist in two types. There are several methods available for long term storage of bacterial stocks from stab plates, and slopes, long-term storing under liquid nitrogen at -196ºC or -80ºC (Prakash *et al.*, 2012).

The first protects against freezing and thawing and the second prevents damage during frozen storage (El-kest and Marth, 1992). This has been observed in various isolates from *Streptococci* spp., *Enterococcus* spp., *Salmonella* spp., *S. aureus* and *Pseudomonas* spp. (Ray and Speck, 1972; Gao and Williams, 2013; El-kest and Marth, 1992; Duval *et al*., 2010). This has mainly been observed in clinical isolates which have lost antibiotic susceptibility, however this project we identified loss of antibiotic resistance within veterinary isolates with unknown efflux pump and resistance gene expression which had been stored for less than 5 years. With storage of the isolates used in this study, isolates were stored without antibiotics within vials of the microbank system (Pro-lab Diagnostics, Austin, Tex.) and kept at -80ºC. The vials come with prefilled cryopreservation solution which states that they should last for 10 years once prepared system (Pro-lab Diagnostics, Austin, Tex.). Stocks were cultured from within 10 minutes of removal and put into -20ºC while out of storage to maintain stock viability. Stocks were placed immediately back into storage to prevent freeze thawing.

Loss of resistance genes has been seen in various bacterial species from long-term cold storage at -80 °C using a microbank system (Pro-lab Diagnostics, Austin, Tex.). The loss of the *mecA* gene in various strains of MRSA which had been stored for over 2 years (Van Griethuysen *et al*., 2005) and the loss of amoxicillin resistance in multiple strains *Helicobacter pylori* (Dore *et al*., 1999) are examples of this. The study by Dore *et al*., 1999 plated the isolates from -80°C storage onto gradient plates MBC to MIC to restore amoxicillin resistance in *H. pylori* isolates which was successful. In this project we attempted a similar resuscitation of lost antibiotic resistance using the re-establishing antibiotic resistance assay on veterinary isolates of *E. coli* and *P. aeruginosa* which was successful in restoring some antibiotic resistance in a number of isolates which had previous changes in their resistance profiles. However instead of using media with gradient concentrations of antibiotics like *Dore et al* (1999) the use of a single concentration of the antibiotics with double exposure through multiple antibiotic exposure assays against the isolates with changes in antibiotic susceptibility showed to be effective in restoring antibiotic resistance profiles in *P. aeruginosa* veterinary isolates compared to *E. coli* veterinary isolates.

Determining a point of exponential growth and efflux pump inhibitors

Growth curves were performed on both NCTC typed strains as well veterinary isolates from BG and environmental locations *of P. aeruginosa and E. coli* to identify an incubation time supplemented with the colony counting. This was done to give a 1-2 x 10^6 CFU/ml with absorbance read at 600_{nm} (Stevenson *et al*., 2016). Colony counting was done to ensure the correct CFU/mL was achieved in line with EUCAST methodology for MIC testing and Iso standard (ISO 20776-1:2019, 2019). The recommended OD identified ranged between 0.4 to 0.6 for both species as

demonstrated by Yang *et al*., 2018 Kobayashi *et al.,* 2006, Agrawal *et al.*, 2019, Ryall et al., 2014 whereby lag phase was identified. The use of colony counting was done to supplement growth curves as there is roughly a 50% margin of error as replicable growth curves are assumed as seen by McBirney *et al* (2016), however the supplementation of colony counting at each OD reading verified the 2.5 hours of incubation time prior to MIC testing to reach exponential growth phase for *P. aeruginosa* and *E. coli* isolates of agricultural isolates and NCTC strains, without continuing the growth curve to stationary phase.

The growth curve was not carried out to stationary phase as assumed exponential phase was obtained to give the CFU/ml indicated by the ISO standard and EUCAST methodology for MIC testing which was subsequently used for the efflux pump inhibitor assay. Using broth cultures allowed for more control when determining a point of exponential growth as only a 10^6 to 10^7 CFU/ml was required, whereas McFarland standard comes in a range from 0.5 - 4, the 0.5 McFarland standard is used for AST as recommended in the EUCAST methodology, and gives 10^8 CFU/ml, by which a higher likelihood of cells being in stationary phase whereby cell replication rate is the same as cell death. This could affect the cellular density in culture broths in addition to changes in protein expressions of cells due to changes in metabolic process as well as cellular function with some studies suggesting transition to a catabolic state (Nyström, 2004). During exponential phase bacterial cells are actively replicating at a constant rate with different metabolic activity and cell surfaces charges compared to stationary phase which could affect the susceptibility of antimicrobials. This was observed with *S. aureus* with changes in levels of methicillin- resistance related to charge and position on growth curve by Matsuo *et al*., 2011. A similar observation was seen in *E. coli* by Agrawal *et al.,* 2019

where variability in permeability was seen of the membrane between mid-log and stationary phase affecting susceptibility. This in turn could change the time taken for incubation prior to E-TEST MIC depending on the position of the broth culture within the growth curve.

Isolates used for E-TEST MIC were randomly selected from the smaller pool of isolates for both *P. aeruginosa* and *E. coli* with one from each location to overcome any choice bias. The addition of cations within the media showed some decreases in MIC however further study is required. The MIC results corroborate the findings with Lamers *et al*., (2013) whereby using the same concentration of EPI reduced the MIC in some of the antibiotic against *P. aeruginosa* wild strains, however the strains utilised in this project held unknown resistant genes and efflux pump activity. The RND efflux pumps were the chosen target for action using PAβN like Lamers *et al* ., (2013) however they targeted the Mex family of RND efflux pumps specifically whereas this study looked at targeting the overall. Cation adjusted Mueller-Hinton media was used to stabilise changes in the membrane of the Gram-negative bacteria due to their supplementation of calcium and magnesium whereas other studies have only used EPIs only like the studies by Lomovskaya *et al*., 2001 and Mawabo, *et al*., 2015.

EPI can be synthetic or naturally occurring. Examples of synthetic are CCCP and PAβN, and natural efflux pump inhibitors such as flavones and isoflavones which are metabolites from natural sources, for example Baicalein which is extracted from thyme leaves (Stavri *et al*., 2006, Mahmood *et al*., 2016). PAβN acts on RND family of efflux pumps as a competitive inhibitor with some antibiotic classes (Pathania *et al*., 2019) in addition to acting on the outer membrane (OM) (Lomovskaya *et al*., 2001) causing a decrease in stability. PAβN interaction with *P. aeruginosa* is

primarily with the MexB, MexD, and MexF pumps and on *E. coli* the AcrAB-TolC efflux pumps (Opperman and Nguyen, 2015). A study by Schuster *et al* (2019) determined that the use of cation adjusted media in addition to higher concentration of PAβN at 25 mg/L didn't change the efficacy of the MIC but with further supplementation of $MgCl_2$ in cation adjusted media gave increased OM stability.

Within this study a range of classes of antimicrobials were utilised (Table 4) with the aim to cover antimicrobials used in both human and agricultural medicine.
The efflux assay shows some preliminary work regarding changes in antimicrobial susceptibility profiles with some of the antimicrobial classes. Ceftazidime showed a decrease in MIC with just the EPI and a greater decrease with the EPI and cation adjusted media against a *P. aeruginosa* isolate from BG (isolate 5). The findings of reductions in MIC of cephalosporins was also seen by Ferrer-Espada *et al* (2019) with wild strain *P. aeruginosa,* however a decrease in MIC in *E. coli* isolates of ENV origin was also seen with the addition of cations or Epi independently. A similar target for ceftazidime resistance was done by Readman *et al* (2016) whereby a clinical strain of *E.* coli was targeted to increase antibiotic sensitivity. All papers used have had specific over expression or induced over expression of efflux pumps such as *Arc* and *Mex* pathways in bacteria investigated whereas the isolates utilised in this project had unknown activation and expression of any of their efflux pumps so EPI interaction would be unknown.

This project looked at the way we determine the susceptibility of veterinary isolate towards antibiotics and whether supplemented AST would alter the course of treatment by reducing the under-representation of antibiotic sensitivity of veterinary isolates.

Testing antimicrobial susceptibility hasn't changed from the gold standard using the disc diffusion method since the 1950's. The main media being used in susceptibility testing is MHA or MHB depending on the method being utilised. MHA contains unknown concentrations of both Mg^{2+} and Ca^{2+} ions, however variation in the concentration of cations present within the media can be seen between different manufacturers (Åhman et al., 2020, Girardello *et al*., 2012). Thus, the same manufacturer was utilised during this project to help limit the variation of cation concentration present within the media. The AST testing undertaken in this study was completed based on the human AST method from EUCAST, due to no preferred AST method within veterinary medicine. Antibiotic breakpoints used in this project were based on isolates from humans as very few breakpoints are available for veterinary isolates; taking this into consideration there are few which have been published by CLSI. These are animal specific and complete lists of antimicrobials and breakpoints for all animal isolates are not available. However, EUCAST V10 (January 2020) has started to incorporate wild strain isolates in the breakpoints as part of the antibiotic stewardship for some of antimicrobials. These are off scale breakpoints with susceptibility recognised with a ZOI ≥50mm.

5. Further Work

Further testing on these veterinary isolates is required using ranges of cation concentration supplementation and EPI as well as, Molecular characterisation of isolates to identify genetic expression of efflux pumps and antibiotic resistance genes and identify any correlation between efflux pump presence and isolate source of origin. Western blot analysis to identify active efflux pumps against different antibiotics at ranges of PAβN concentrations to identify changes in susceptibility, in

addition to further supplementation with a range of different cations at various concentrations. The use of alternative media for isolate growth and minimal media for MIC and efflux assays. Further E-TEST MIC testing all available veterinary isolates which have been purified and stored. Performing MIC and AST at varying stages of bacterial growth curve on the isolates to identify changes in susceptibility and maturity, in addition to biofilm formation and efflux pump activity within biofilms. MIC broth microdilution could also be beneficial to run using this and various other efflux pumps, such as CCCP which has been seen to reverse colistin resistance in Gram-negative bacteria using the microbroth dilution method (Baron and Rolain, 2018).

6. Conclusion

This study focused on whether the presence of cations in AST media would cause a significant difference in the antibiotic susceptibility of veterinary isolates and started to identify any impact caused by inhibitory substances which would causes changes in the isolate susceptibility. Both *P. aeruginosa* and *E. coli* NCTC typed and veterinary isolates showed similar antibiotic susceptibility profiles being established to the previous student, however changes in antibiotic resistance profiles of some of the isolates was identified. The main change identified was loss of antibiotic resistance, however the restoration of the antibiotic susceptibility profiles was achieved with some increases in resistance emerging with noted β-lactam and tetracycline resistance restored through an adaptation of gradient agar method with single antibiotic concentration exposure through multiple assays to those isolates with changes identified. An estimate of exponential phase growth was identified at 2.5 hours with colony counting supplementation, although some variation was seen between both species and individual isolate growth curves. The preliminary data for

E-TEST MIC showed some decreases in MIC value of some of the veterinary isolates towards the representative antibiotic of carbapenem and cephalosporin with the presence of cation adjusted media and PAβN together and independently. Further testing is required with this EPI to confirm changes in antibiotic susceptibility of the veterinary isolates in addition to testing the NCTC typed isolates and determine MIC changes for a larger proportion of veterinary isolates and species available.

7. References

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