Antimicrobial resistance in indicator bacterial species from wildlife at the human-livestock-wildlife interface in the Mnisi community, Mpumalanga, South Africa

By

Lawrence Mugochi

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Supervisor: Professor Vinny Naidoo
Co Supervisor: Dr Annelize Jonker

DECLARATION

I Lawrence Mugochi, declare that this dissertation that is hereby presented to the University of Pretoria for the Master of Science (Veterinary Science) degree is my own work and it has not been presented for any other qualification or award in another institution. I have acknowledged and referenced all secondary material used as required by the University of Pretoria.

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- The Almighty for ability and provision.

ABSTRACT

Antimicrobial resistance (AMR) is one of the greatest threats currently facing humanity. While there have been extensive studies on this subject in public health and livestock, there is paucity of information on the epidemiological role of wildlife in AMR. Anthropogenic activities have been suggested to be the main reason for presence of AMR in wildlife. Wildlife can also be a reservoir of naturally occurring resistance. While a one health approach has been put in place to tackle AMR, there have been obstacles in implementing it.

The aim of this study was to determine the resistance of *E. coli* and *Enterococcus* isolates from wildlife faecal samples. Fifty-one wildlife faecal samples (herbivores and carnivores) collected from Mnisi between 2015 and 2020 and stored in a biobank at Hans Hoheisen Research Station were used in this study. Isolation of *E. coli* and *Enterococcus* was done on MacConkey and Blood agar and Biochemical tests done to identify isolates. Minimum inhibitory concentration was done using Thermo Scientific Sensititre plates according to manufacturer recommendations and interpreted using Clinical Laboratory Standards Institute standard tables.

A total of 14 *E. coli* and 42 *Enterococcus* isolates were obtained. 52% of the Enterococcus isolates were identified as *E. faecium* and 48% as *E. faecalis*. Antimicrobial susceptibility analysis for *E. coli* showed one multidrug resistant isolate, 14% susceptibility to cefuroxime and 7% susceptibility to cefazolin, cefepime, ceftriaxone, cefoxitin and cefpodoxime and imipenem. Antimicrobial susceptibility for *Enterococcus* revealed 42% of isolates had multi drug resistance. Daptomycin, Rifampin and Quinuptistin/dalfopristin had highest resistance of 71%, 64% and 43%

respectively. The proximity of the wildlife to livestock and human settlement is likely to be related to the high level of resistance as sewage wastes, run off water and environmental contamination may be responsible for the dissemination of resistant genes. The results of this study show that wildlife is a major player in antimicrobial resistance. The researchers recommend follow up studies and AMR surveillance in wildlife, human hospitals, livestock and water bodies in the area.

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LIST OF ABBREVIATIONS

AAC	acetyltransferase
ABC	ATP-binding cassette family
AMR	Antimicrobial resistance
ANT	adenyl transferase
APH	phosphotransferase
CLSI	Clinical and laboratory Standards Institute
DNA	Deoxyribonucleic Acid
DVTD	Department of Veterinary Tropical Diseases
E. coli	*Escherichia coli*
EUCAST	European Committee on Antimicrobial Susceptibility Testing
HHWRS	Hans Hoheisen Wildlife Research Station
KNP	Kruger National Park
MFS	Major Facilitator Superfamily
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin Resistant *Staphylococcus Aureus*
mRNA	messenger RNA
PBP	Penicillin Binding Proteins
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
RND	The resistance-nodulation-cell-division

SMR	Small multidrug resistance family
tRNA	transport RNA
WHO	World Health Organization
WOAH	World Organisation of Animal Health

CHAPTER 1: INTRODUCTION

Antimicrobial resistance occurs when bacteria, parasites and viruses evolve and acquire mechanisms that shield them from the effects of chemicals that could potentially kill them or prevent their growth, including medicines. In 2020, the World Health Organisation (WHO) declared antimicrobial resistance as part of the 10 global public health threats affecting humanity (WHO 2020). The WHO further stated that urgent multisectoral action is required to mitigate the impact of being unable to treat infections, if the world is to achieve the Sustainable Development Goals. While the use of antimicrobials in humans and livestock is probably the most important source of antimicrobial resistance, focusing on these species alone can give an incomplete epidemiological picture (Allen et al. 2010).

The soil and other natural habitats can serve as reservoirs of naturally occurring antimicrobial resistance (Arnold et al. 2016). Shared grazing lands and proximity of wildlife to humans and livestock at human livestock wildlife interfaces allow access of wildlife to human and livestock waste. This can result in transfer of resistant microbes between these species (Allen et al. 2010). Wildlife will in turn disseminate these microbes to distant areas and the wildlife species can end up being a source and reservoir of resistant microbes. As examples, Mariano (2007) reported 9.95% antimicrobial resistance to tetracycline in impala around the Kruger National Park (KNP), while Jobbins and Alexander (2015) reported a 41.3% antimicrobial resistance in 18 different wildlife species in Chobe, Botswana. In Nairobi Hassell et al. (2019) found 52% multidrug resistance to *Escherichia coli* in 75 wildlife species. Further, Vittecoq et al. (2009) compared various studies and concluded that

carnivores and omnivores are more likely to have antimicrobial resistance compared to herbivores, likely due to differences in their native microflora.

In South Africa, many communities reside adjacent to the Kruger National Park (KNP) and as a result cattle, people and wildlife often interact. One such area is the Mnisi community of the Bushbuckridge municipality in Mpumalanga province which lies in close proximity to the KNP, but also a few other game reserves. In the Mnisi district Mupfunya et al. (2021) previously reported that 27% of *E. coli* isolates from cattle faeces were resistant to various antibiotics. They also reported resistance to colistin which is a last resort antimicrobial drug used in humans. It is therefore important to investigate if Mupfunya's findings are similar in the wildlife species at the interface, to understand the actual spread of resistance in the area, using *E. coli* and *Enterococcus* spp., as recommended by the World Organisation for Animal Health (WOAH) (WOAH 2020).

1.1 Aim

To isolate *E. coli* and *Enterococcus* species from bio banked wildlife faecal samples harvested near the Mnisi community and to determine the susceptibility of the isolates to selected antibiotics.

1.2 Objectives

- To isolate *E. coli* and *Enterococcus* species from faecal samples collected from wildlife droppings in the area.

- To determine the susceptibility of the isolated bacterial species to selected antibiotics.

1.3 Benefits of the research project

- It will give baseline information on antimicrobial resistance in wildlife in Mnisi town.
- It will add insight on the role of wildlife in the prevalence of antimicrobial resistance.
- The results can be used as a guide for policy makers on antimicrobial use and wastewater/sewage management. It can also outline the importance of the implementation of the AMR strategic framework in South Africa.

1.4

CHAPTER 2: LITERATURE REVEIW

2.1 History and action of antimicrobials

Diseases can be classified into infectious or non-infectious diseases, with infectious diseases being diseases caused by micro-organisms. They account for a large proportion of diseases in both humans and animals. The discovery that many diseases that affect man were caused by microorganisms was first made in the later parts of the nineteenth century (Saga & Yamaguchi 2009). The discovery of antimicrobials followed thereafter as an invaluable means of controlling disease, when Ehrlich discovered the first recorded antimicrobial substance in 1910. The drug was called salvarsan and it was used for the treatment of syphilis. Salvarsan was made from a toxic arsenic compound which Ehrlich modified to an organo-arsenic derivative so that it can be more selectively toxic to the pathogen (Lloyd et al. 2015). The next antibiotics to be discovered were sulphonamides which were developed in 1935. Both antimicrobials were synthetic and had issues with safety and efficacy (Saga & Yamaguchi 2009).

In 1928 Fleming accidentally discovered that a fungal contaminant (Penicillium) inhibited the growth of *Staphylococcus* bacteria (Saga & Yamaguchi 2009). This eventually led to the development of penicillin which entered into mainstream use after 1940. According to Saga and Yamaguchi (2009), penicillin had an excellent safety and efficacy profile and was so widely used in the treatment of wounded soldiers in the second world war that it sparked the antimicrobial revolution. This was evident over the next two decades and which saw the discovery of other classes of antimicrobials, including streptomycin, chloramphenicol and the tetracyclines from

soil bacteria, and which lead to the golden era of antimicrobials (Saga & Yamaguchi 2009). The development and modification of previously identified antimicrobials also improved the spectrum of antimicrobials as they could target a large variety of organisms and in some cases specific organisms.

Unfortunately, antimicrobial resistance followed as soon as these antimicrobials entered into common use. The Food and Agricultural Organisation (2022) defined antimicrobial resistance as the capability of microorganisms to grow in the presence of drugs developed to inhibit said growth or kill them. Typical examples include *Staphylococcus aureus,* which developed resistance to sulphonamides shortly after the antimicrobial was discovered. As early as the 1950s, strains of the bacteria were shown to produce the enzyme penicillinase which had the ability to break down penicillin, rendering the said bacteria resistant to penicillin (Saga & Yamaguchi 2009). As a result, further interventions were required, which included modification of the penicillin antibiotics so that the drug was not as susceptible to the penicillinase enzyme e.g methicillin was introduced in 1959. Unfortunately, even this was not sufficient as in 1961 methicillin resistant *Staphylococcus Aureus* (MRSA) was isolated (Jevons 1961).

The action of antibiotics is through targeting the structure and/or metabolic pathways of the bacterial cell. According to Etebu and Arikekpar (2016) the mechanisms can be summarised as follows:

- Inhibition of cell wall synthesis: Bacteria have a rigid cell wall on the exterior surface, made up of peptidoglycan crosslinked with peptide bonds. Enzymes called Penicillin Binding Proteins (PBP) are involved in the crosslinking of the

peptidoglycan. Inhibition of the PBPs (penicillins, carbapenems and cephalosporins) or binding to the precursor molecules (vancomycin) can result in bacterial failure in the cell wall and death of the cell (Kahne et al. 2005; Park & Uehara 2008).

- Breakdown of the cell membrane: Different groups of antibiotics cause breakdown of the cell membrane by different mechanisms, e.g. daptomycin depolarizes the calcium-dependant membrane which results in disruption of the membrane and cessation of synthesis of other macromolecules (Alborn et al. 1991), while polymyxin antibiotic binds to the lipid part of lipopolysaccharide on the cell membrane thereby disrupting it (Falagas et al. 2010).

- Inhibition of protein synthesis: The bacterial 70S ribosome is made up of a 30S and 50S subunits. The antibiotics that inhibit protein synthesis do so by either inhibiting the 30S or 50S sub unit causing misreading of mRNA and termination of translation (aminoglycosides) (Kapoor et al. 2017). This inhibition can occur at different points such as inhibiting the 30S subunit and blocking the access of aminoacyl-tRNAs to the ribosome (tetracycline), inhibiting the 50S through inhibiting the initiation of protein synthesis (oxazolidinones) or the elongation (macrolides) phases of translation (Etebu & Arikekpar 2016).

- Inhibition of nucleic acid structure and function: The disruption of nuclei acid synthesis in bacteria will result in the death of the cell as nucleic acids are

essential for life. Antimicrobials interfere with synthesis by either blocking replication or transcription of DNA (Etebu & Arikekpar 2016) or by inhibiting the gyrase or topoisomerase enzyme (quinolones)(Kapoor et al. 2017).

- Blocking metabolic pathways: Sulphonamides and trimethoprim inhibit different steps in folic acid metabolism in bacteria, by competitive inhibition of dihydropteroate synthase (sulphonamide) or through inhibition of the enzyme dihydrofolate reductase (trimethoprim)(Kapoor et al. 2017). Since the two drugs work on subsequent steps in folic acid synthesis, combining the sulphonamides with trimethoprim results in a synergistic effect (Kapoor et al. 2017).

2.2 Antimicrobial resistance

Bacteria have the ability to survive in the environment because they have genetic plasticity which enables them to withstand environmental threats, including the presence of antimicrobials (Munita & Arias 2016). Such bacteria have evolved to resist harmful effects of the antimicrobials and this intrinsic resistance allows them to live in this environment (Munita & Arias 2016). Also, of importance is that a number of developed antimicrobials are derived from soil fungal organisms (natural) and as such have lived in the same ecological niche with bacteria and have adapted mechanisms against these drugs (Munita & Arias 2016). While there are different mechanisms by which resistance developed, the underlying resistance generally develops from either mutation or acquired foreign DNA which codes for resistance (horizontal gene transfer).

2.2.1 Mutation

In the presence of antimicrobial pressure, part of the susceptible bacterial population tends to undergo genetic mutations which affects the ability of the antimicrobial to function, resulting in the survival of that mutant. Since the antimicrobial will eliminate the susceptible population, the mutant population will eventually predominate. Examples of the effect of the mutation on the antimicrobial activity can either be:

- Modification of antimicrobial target site(s), reducing the affinity of the antimicrobial for its target site, with the result that the drug is no longer able to function by its defined mechanism of action. Modification of antimicrobial targets can be a result of a single point mutation or multiple point mutations, changing the amino acid composition of the target site (Gumbo 2011).
- Decrease in antimicrobial uptake: Gram-negative bacteria have a selective barrier on their outer membrane which prevents large and polar molecules from entering. Antimicrobials which are small and polar make use of channels called porins to enter the cell (Gumbo 2011). A mutation of the genes coding for the porins can slow down the rate of a drug entering the cell.
- Activation of efflux mechanisms to remove the antimicrobials: Microorganisms have efflux pumps that expel unwanted materials from the bacteria. Mutations can result in overexpression of these efflux pumps. This can result in rapid expulsion of drugs, making the drugs less effective. A good example is the resistance of the malaria parasite to chloroquine which is due to a mutation in the gene coding for the ABC efflux pump (Gumbo 2011); or

- Development of alternative metabolic pathways (Munita & Arias 2016): This results when the bacteria use alternative pathways to produce essential metabolites.

2.2.2 Horizontal gene transfer

This is a very important driver of antimicrobial resistance. Bacteria that already possess resistance genes are able to transfer the genes to other bacteria, including to clinically relevant strains (Munita & Arias 2016). Horizontal gene transfer occurs through transformation, transduction or conjugation. Transformation is when naked DNA, carrying resistance, is incorporated into the bacterial genome. This is a rare phenomenon. Transduction is through a bacteriophage (viral particles that infect bacteria and transfer genetic material) . Conjugation involves bacterial cell to cell contact with transfer of mobile genetic elements. Mobile genetic elements include plasmids, transposons and integrons (Manson et al, 2010):

- Plasmid: A plasmid is a small extrachromosal DNA that can replicate independently. They are circular DNA molecules. They are not critical for the bacterial survival, but they may 'confer a selective advantage', for example antimicrobial resistance (Britannica 2022).
- Transposon: A transposon is a DNA sequence that can alter its position (jumping DNA) within a genome thereby altering the cell's genetic identity (Bourque et al. 2018). They normally contain repeating nucleic acid sequences at their ends to facilitate their excision from the sequence. Once they are cut, they can re-enter the genome at a different position. This can alter genome expression.
- Integron: Also a very efficient ways of accumulating antimicrobial resistance. These are 'site specific recombination systems that are able to integrate,

express and exchange specific DNA elements, called gene cassettes' (Munita & Arias 2016). They are composed of the gene that encodes the integrase enzyme that is required for recombination, the recombination site specific for the integrase and a promoter that is necessary for expression of the gene cassette (Domingues et al. 2012). They are very efficient in adding new genes into chromosomes.

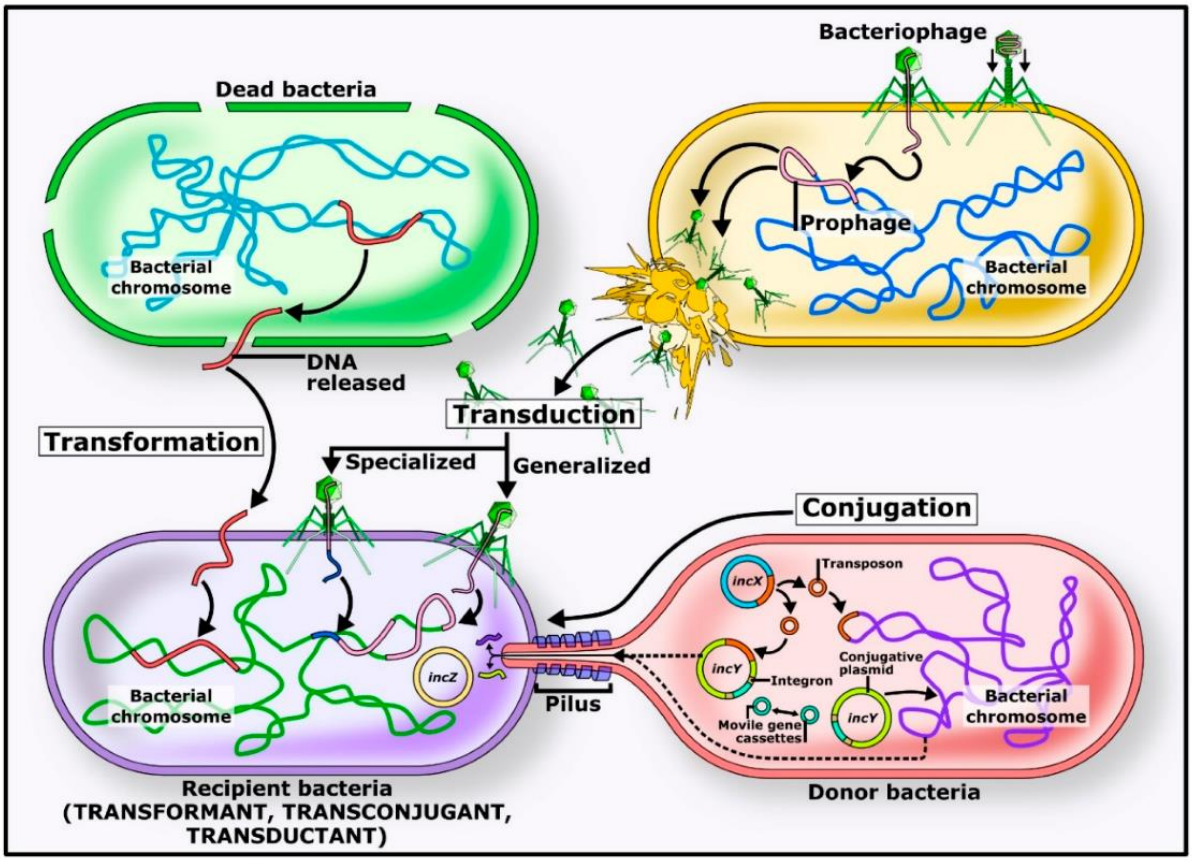

Figure 2.1: Illustration of different mechanisms of horizontal gene transfer (Bello Lopez, 2019).

2.2.3 Mechanistic basis of antimicrobial resistance

While various mechanisms of resistance have been described, according to Munita and Arias (2016), the general mechanisms of antimicrobial resistance development by bacteria can be classified into four broad categories. These are modification of the antimicrobial molecule, prevention of access to the antimicrobial target, changes or bypass of the target site and global cell adaptive processes.

2.2.3.1 Modification of antimicrobial molecule

In this type of the resistance, the organisms have mechanisms to render the antimicrobial ineffective as a chemical. This may occur when the bacteria produce enzymes which either destroy the antibacterial molecule or causes alterations to the molecule. β-lactamase enzyme is a typical example of this effect. The enzyme is produced by resistant bacteria and destroys the amide bond on the β-lactam ring in β lactam antimicrobials e.g. penicillin (D'Costa et al. 2011). While numerous bacterial species have been shown to produce the enzyme, *S. aureus* is the most common and important example.

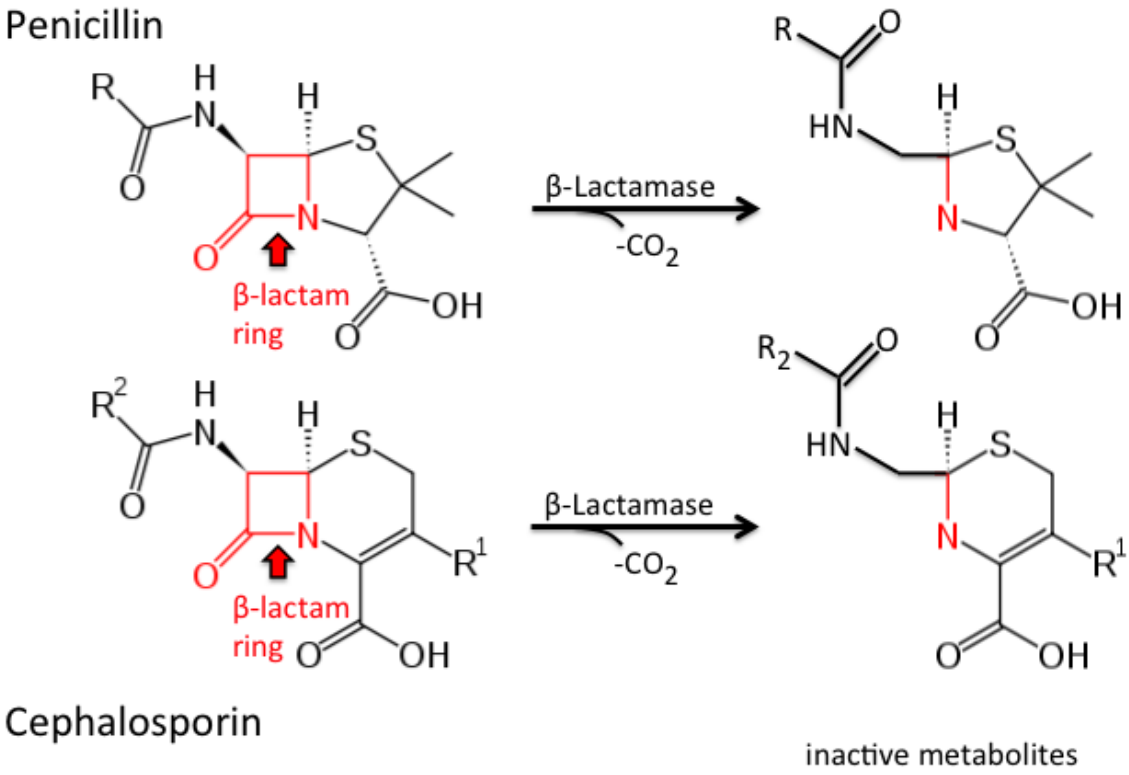

Figure 2.2 Illustration of the hydrolysis of the β lactam ring by β lactamase in penicillin and cephalosporins (TMedweb 2015).

Some bacteria are able to produce enzymes that modify antibacterial molecules. This is common in antibacterial agents which inhibit protein synthesis at ribosome level (Ramirez & Tolmasky 2010). Modification can be by acetylation, phosphorylation or adenylation reactions. Modification results in reduced affinity of the antibacterial molecule for its target due to a steric hindrance. An example of enzyme modification is the Aminoglycoside Modifying Enzymes which covalently bind the amino or hydroxyl group of aminoglycosides (Ramirez & Tolmasky 2010). The genes encoding these Aminoglycoside Modifying Enzymes are normally found on mobile genetic elements but they can be part of the bacterial chromosome, as seen with *Enterococcus faecium*.

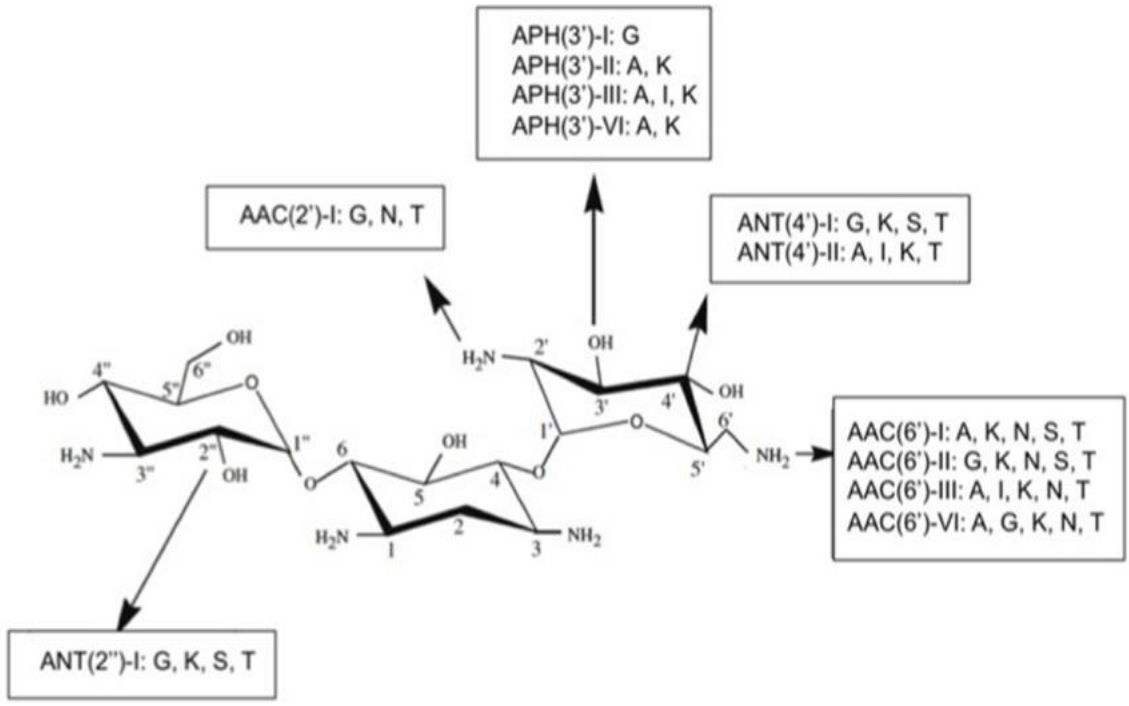

Figure 2.3 Aminoglycoside Modifying Enzymes and their sites of action. They are named by their biochemical mode of action, that is acetyltransferase (AAC), adenyl transferase (ANT) and phosphotransferase (APH) (Munita & Arias 2016).

2.2.3.2 Prevention of access to the antibacterial target

Antibacterial drugs can be prevented from reaching their target either by decreasing permeability of the antibacterial into the bacterial cell or by increasing activity of efflux pumps that remove the antibacterial from the cell. Most targets for antibacterial drugs are intracellular or on the inner membrane of Gram-negative bacteria. In Gram-negative bacteria, hydrophilic antibacterial drugs like β-lactams need porins (proteins that establish the transmembrane channels) to enter the cell as they cannot pass through the cell membrane. Impaired porin function or reduced expression of porins will thus result in reduced uptake of the antibacterial. Mutations and aberrant production of OprD, the porin on *Pseudomonas aeruginosa,* have been associated

with resistance of the organism (Quinn et al.1986). Another clinically relevant example is the shifting of porin expression from OmpK35 to OmpK36 in *Klebsiela pneumoniae* after exposure to antimicrobials (Hasdemir et al. 2004). This reduces penetration of β-Lactam antibiotics.

Bacteria also have complex efflux pumps to remove toxins and these can also remove antibacterial compounds. Five classes of efflux pumps have been documented in bacteria. These are the major facilitator superfamily (MFS), the small multidrug resistance family (SMR), the resistance-nodulation-cell-division family (RND), the ATP-binding cassette family (ABC) and the multidrug and toxic compound extrusion family (MATE) (Piddock 2006). An example of this type of resistance is tetracycline resistance. The Tet efflux pump, which belongs to MFS, extrudes tetracycline in exchange of a proton limiting the ability of the drug in interfere with protein synthesis (Poole 2005).

2.2.3.3 Changes to or bypass of the target site

Antimicrobials are able to function by binding to selected targets within the bacterial cells to bring about their effect. The main target sites for the antimicrobials are the cell wall, cell membrane, protein synthesis, RNA synthesis, DNA synthesis and transporters.

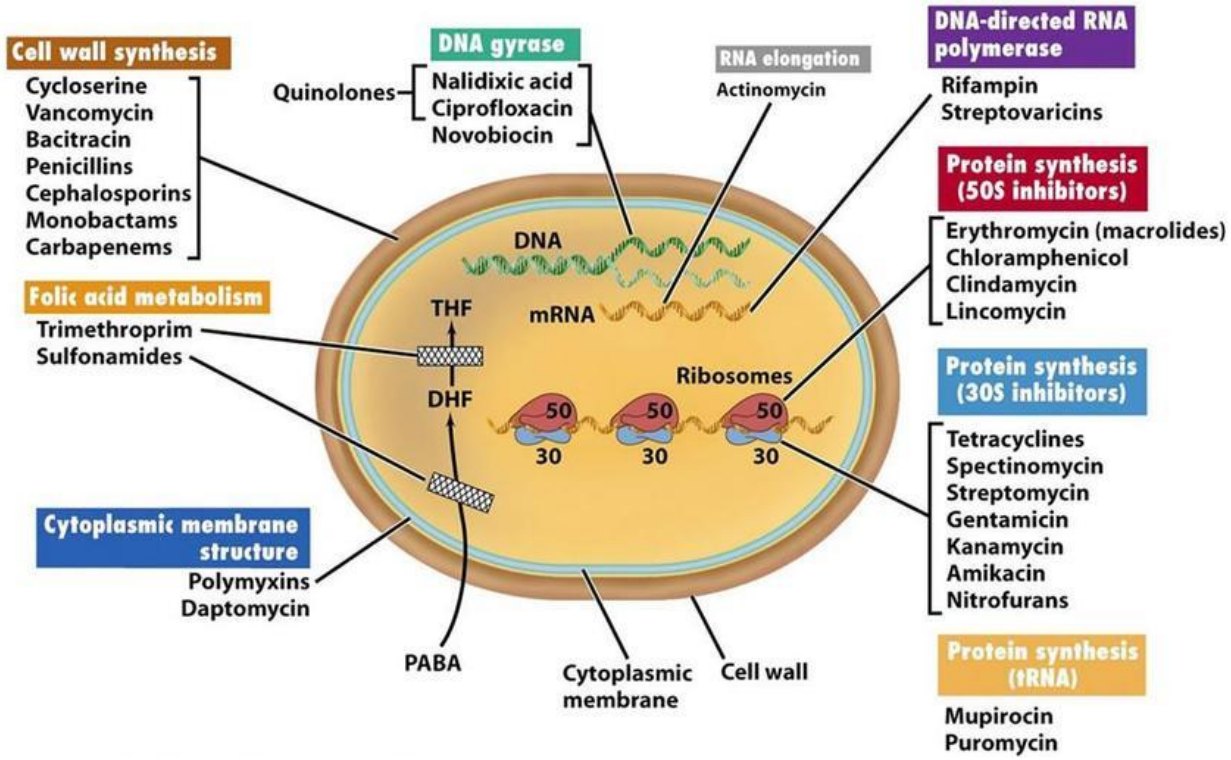

Figure 2.4: Antibiotic target sites of the common antimicrobial agents in use (Madigan & Martinko 2006)

Bacteria have evolved mechanisms to protect the target sites from antibacterial drugs or to modify them to become less susceptible to the antibacterial. Genes encoding target protection are mainly located on mobile genetic elements. Tetracycline resistance determinants TetM and TetO (Tet is a ribosomal protective protein) interacts with the ribosome and dislodges the antibiotic from the binding site, thereby protecting the target site (Connell et al. 2003). Another important example is the Qnr fluoroquinolone resistance determinant. Fluoroquinolones kill the bacterial cell by forming a complex with the DNA gyrase. The Qnr resistance determinant acts as a DNA homologue and competes for the binding site on DNA gyrase and

topoisomerase IV, decreasing the ability of the antibiotic to complex and kill the cell (Rodríguez-Martínez et al. 2011).

Modification of the target site is a very common mechanism of antimicrobial resistance. This may arise as a result of either point mutations of genes encoding for the target site, bypassing the target site or using enzymes that alter the target site (Munita & Arias 2016). An example of such a point mutation is the development of rifampin resistance. Rifampin acts by blocking DNA dependant RNA polymerase, thereby preventing transcription. A point mutation in the gene which encodes the binding pocket of rifampin on the RNA polymerase decreases the affinity of the antibiotic for the site, without changing the catalytic activity of the enzyme (Campbell et al. 2001).

An example of alteration of the target site with enzymes is seen in macrolide resistance. As described above, macrolides inhibit protein synthesis by binding to the 50S ribosome subunit. A resistance gene called the Erythromycin Ribosomal Methylation gene encodes an enzyme which causes methylation of the ribosome. This will in turn impair the binding of the macrolide antibiotics to the ribosomal complex, conferring resistance (Leclercq 2001). The gene is present in mobile genetic elements.

Some bacteria are able to bypass/ replace targets for antimicrobials. In this way bacteria are capable of evolving a different target which is able to perform similar biochemical processes to the original target but which are not susceptible to the antibiotic molecule. This mode of resistance is seen in methicillin resistance in

Staphylococcus aureus. β lactams act by disrupting cell wall synthesis by binding to Penicillin Binding Proteins (PBP). Resistance to methicillin results from the bacteria acquiring a foreign gene which encodes PBP2a. PBP2a is a penicillin binding protein with low affinity for β lactams, that has the same function as PBP in the bacteria (Munita & Arias 2016).

2.3 Determining the presence of resistance

One of the problems with bacterial infections is the inability to macroscopically visualise the agents causing an infection and thus linking the agent to the optimal antimicrobial agent. As a result, the medical profession has resorted to the empirical use of antimicrobials. With this form of antimicrobial selection, one typically uses the broad-spectrum antibiotics to treat infections without having knowledge of which bacteria is causing the infection in the hope of targeting any bacterial agent that may be present. While initially effective, this has resulted in overuse of certain antimicrobials which has resulted in the appearance and spread of multi resistance organisms (Petersen et al. 2019). For more prudent manner of selection, guidelines were developed to provide the treating physician with a likely distribution of organisms in specific tissues. Through this principle, a more intermediate spectrum drug could be used. However, in time, as with the use of the broad-spectrum drugs, resistance is also promoted. As a result, the most prudent practice is to identify the causative organism through culture and susceptibility testing. Various methods are currently available for the said testing:

2.3.1 Dilution methods

These methods are used to establish the minimum inhibitory concentration (MIC) of antimicrobial agents being tested for. The MIC is the lowest dose that cause the inhibition of bacterial growth under laboratory conditions. At present, the method can either be done on broth (broth macrodilution or microdilution) or on agar (agar dilution). The guidelines for the dilution methods have been standardised to ensure reliable use in any laboratory. The standards that are commonly used are the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute (CLSI). EUCAST and CLSI deal with breakpoints and other relevant technical aspects of antimicrobial susceptibility testing. The dilution methods can be divided further into the following

- Broth macrodilution method: This involves incubation of two-fold dilutions of the antibiotic or antifungal agent in a broth medium containing 2ml of the standardised microbial suspensions adjusted to 0·5 McFarland turbidity scale. Bacteria are incubated at 37^{o}C for 24 hours and fungi at 25^{o}C for 4-10 days. (Balouiri et al. 2016) The tubes will be observed for turbidity which indicates growth. The MIC is the lowest antimicrobial concentration to inhibit growth. The major advantage of this method is that it provides quantitative results however it has the disadvantage of requiring large space. it is also labour intensive.

- Broth microdilution method: This method uses a plate containing wells of volume of about 0.1-0.2ml. Each plate contains about 96 wells and can test multiple antibiotics (12) over a range of eight two-fold dilutions. The wells are

filled with serial two-fold dilutions of the antibiotic agent and inoculated with the microbial suspension after it has been standardised to 0.5 McFarland turbidity to reach a concentration of 1-5 x 10^5 cfu per ml (Brook et al. 2013). The plate is incubated as in the macrodilution method. The MIC is read manually or using an automated viewing device. The advantage of this method is that it requires a small sample size and is less expensive. The disadvantage is that it is difficult to detect contamination.

- Agar dilution: This method involves the incorporation of serial two-fold dilutions of antimicrobial agent onto molten agar followed by inoculation of standardised test bacterium (Ge et al. 2013). Likewise, the MIC is the lowest concentration to inhibit growth. The advantage of this method is that many bacteria can be tested on the same plate. The major disadvantage is that the process is not semi-automated and is labour intensive.

2.3.2 Agar disc diffusion method

This is one of the oldest methods developed and still in use. Standardised microorganism is inoculated onto Mueller–Hinton agar plates and discs containing antibacterial at the desired concentration are laid on the agar surface and incubated at 35-37°C for 24 hours (EUCAST 2019). The diameter of inhibition of growth around each disc is measured. The results are qualitative and can either be susceptible, intermediate or resistant. The main advantage of this method is that it is simple and relatively cheap.

2.3.3 Antimicrobial gradient method

This method combines the principles of diffusion and dilution. The agar plate is inoculated with the test bacterium and a strip impregnated with increasing concentrations of dried antibiotic is placed onto the agar surface (Wiegand et al. 2008). After overnight incubation, the point of intersection of the strip and zone of inhibition is the MIC.

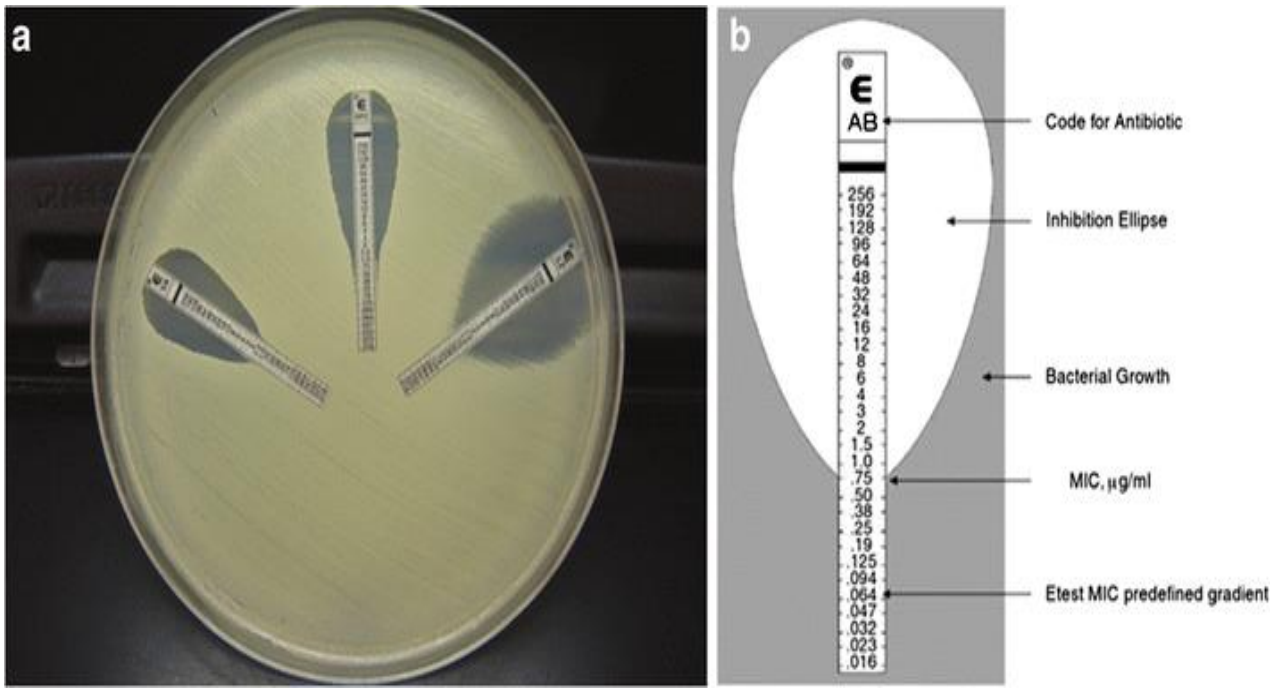

Figure 2.5 Illustration of antimicrobial gradient method (Schumacher et al. 2018).

2.3.4 Polymerase Chain Reaction based Methods

These have been developed to detect genetic determinants of specific antimicrobial resistance in test isolates (Fluit et al. 2001). A good example of this is the detection of mecA gene in MRSA. MecA encodes for an alternative protein PBP2b which has a lower affinity for β-lactam antibiotics than the original Penicillin Binding Proteins. Other examples include detection of carbapenamase encoding genes and vanA and vanB genes for vancomycin resistance in *Enterococcus* (Cekin et al. 2013). PCR

based methods are in general fast and efficient, such that results can be obtained in as little as two hours.

While antimicrobial susceptibility testing has enhanced the responsiveness of antimicrobial therapy, the methods routinely used for antimicrobial susceptibility testing have a drawback of being time consuming. It generally takes a day to two to grow the bacteria and an additional day to characterise and identify the isolate. While the development of commercial automated devices like the Microscan (Beckman Coulter) (Benkova et al. 2020), which use optical systems to detect minute changes to determine turbidity (growth), allow for turn-around times of as low as 6 hours, they too are problematic. The major drawbacks include high costs, limited spectrum of microorganisms in the database and requirement of large numbers of viable microorganisms (van Belkum & Dunne 2013). Real time Polymerase Chain Reaction (PCR), mass spectrometry, flow cytometry and microarrays have been developed and the major challenge with these is with costs and that they require technically qualified staff (Miller & Tang 2009).

2.4 Antimicrobial Resistance and wildlife

Antimicrobial resistance has caused a significant impact on the world economy and human and animal health. AMR results in increased hospital stay, higher medical costs and increased death rates. It has been estimated that deaths related to antimicrobial resistance are now more than HIV and malaria related deaths (Cox 2022). In addition to increasing human mortalities, there is now evidence which shows the occurrence of antibiotics and antimicrobial resistance in wildlife within natural environments (Allen et al. 2010). Jobbins and Alexander (2015) reported a

41.3% antimicrobial resistance in different wildlife species in Botswana. In Nairobi, Hassell et al (2019) found 52% multidrug resistance in *E. coli* in wildlife from different species.

As a result, it is very important to understand the role of humans, domestic animals, wildlife and the environment in the sustenance and dispersal of resistance in bacterial populations (Vittecoq 2016). At present, there are extensive studies in the human population and domestic animals to understand how resistance develops. Despite it being known that antimicrobial use in animals can impact the environment, the dynamics of resistance in wildlife is not yet fully understood.

There are many possible avenues of transfer of resistant bacteria between wildlife, humans and domestic animals:

- Consumption: At the wildlife, domestic animal and human interface, wildlife can feed on dead domestic animals which can harbour resistant bacteria (Vittecoq 2016). While not quantified, it is just as likely that humans will also be exposed to resistant bacteria on poorly prepared game meat.
- Water: Resistant bacterial isolates have been isolated from rivers, lakes and sea water ((Zhao & Dang 2012). *Salmonela, E. coli, Staphylococcus and Klebsiella* species are known to persist in water for long periods of time (Goodwin et al. 2012). The bacteria can originate from human and domestic animal populations. Sewage wastes are not completely free of bacteria and end up in rivers. Droppings of livestock can contaminate the environment and ultimately end in rivers. Aquaculture practices also involve the use of large

amounts of antibiotics and most of the left-over antibiotics and organic matter end up in nearby water bodies (Cabello et al. 2013; Buschmann et al. 2012).

- Soil: The source of the resistance can either be intrinsic or due to faecal and urine contamination from livestock or human effluent flows (Wellington et al. 2013).
- Wind: Allen et al. (2011) suggests aerial dispersal as another route of transmission. Wind can disperse antibacterial particles as well as resistant bacteria.

The wildlife habitats that are close to the human livestock interface are likely to be the most contaminated by resistant bacteria (Allen et al. 2010). Beyond this, how the wildlife species eat and drink and their movements determine the spatial distribution of the resistance bacteria. Studies already completed suggest that carnivores and omnivores carry more resistance bacteria compared to herbivores. This may be related to their diet and also the fact that herbivores have the highest microflora diversity compared to carnivores and omnivores (Ley et al. 2008). Predators and migratory birds also have the greatest capacity to disperse resistant bacteria since they travel long distances (Middleton & Ambrose 2005).

While there is paucity of information on wildlife resistance in indigenous South African animals, a recent study in Mnisi community reported that 55% of *E. faecalis* isolates from cattle were resistant to enrofloxacin and 3% resistant to amoxicillin (Mupfunya et al. 2021). The study also reported that 16% of *E. coli* species were resistant to colistin, 8% to chlortetracycline and 8% to amoxicillin. An important feature of this specific community is that it is situated in Bushbuckridge Municipal

Area, Mpumalanga Province, South Africa, with approximately 75% of the community bordered wildlife conservation areas including the Great Limpopo Transfrontier Wildlife Conservation Area. With about two-thirds of households in Mnisi owning livestock (Berrian et al. 2016), it is plausible that resistance could spread to wildlife species in the area. This study aims to investigate the trends of resistance in wildlife in the same area and to establish if resistance in cattle strains is being transferred.

2.5 **Combating AMR**

As seen above, AMR is an ecological problem which involves complex interactions of microbials which can affect humans, animals and the environment. It would make sense to use a one health approach in tackling AMR. The One health approach is a 'collaborative multisectoral approach to achieve optimal health by recognising that there is an interconnection between humans, animals, plants and their environment' (CDC 2022). In 2015 the World Health Assembly (WHO, FAO, WOAH) adopted a global action plan on antimicrobial resistance, with the following objectives:

- To improve awareness of AMR through training and communication.
- To increase surveillance and research in order to strengthen knowledge and evidence.
- To promote sanitation, hygiene and other preventative measures in order to reduce incidence of infection.
- To optimise the use of antimicrobials in human and animal health.
- To develop the economic case for sustainable investment that takes account of the needs of all countries and to increase investment in new medicines, diagnostic tools, vaccines and other interventions (WHO 2016).

In South Africa a national AMR strategic framework was established in 2014 (Shabangu et al. 2023), and has a Ministerial Advisory Committee which coordinates and implement the framework. However there have been many challenges in the implementation of the strategic framework. Shabangu et al (2023) raised concerns as there is no dedicated budget, insufficient human resources to implement the framework, or a formal AMR surveillance system in place. Shabangu et al (2023) further highlighted that the availability of over the counter antibiotics for use in livestock is another barrier to the success of the strategic framework, as Act 36 of 1947 allows for certain antimicrobials used in livestock to be sold as over the counter drugs. As a result, until these are addressed it is unlikely that South Africa would properly addressed current concerns with AMR.

CHAPTER 3: MATERIALS AND METHODS

3.1 Study population and sample collection

Faecal samples (n=51) from wildlife species opportunistically collected by vets between 2015 and 2020 in the area and stored at the HHWRS biobank at -80°C were used for this study. The samples were selected by convenience. Only 51 samples could be used due to sample availability. Swabs of faecal material were taken from the samples in the biobank in May 2022. Utmost care and precautions were taken to prevent cross contamination of the samples. Swab samples so collected were labelled and maintained in sterile, sealed leak-proof transport media containers to preserve organisms. This primary packaging was further maintained in a clean secondary water-tight leak-proof container. The secondary package carrying samples was conveyed in a cooler box carrying ice packs to the Department of Veterinary Tropical Diseases Bacteriology Laboratory. Once at the laboratory, the secondary package carrying the samples was immediately moved into the -80°C freezer for storage pending analysis.

3.2 Bacterial isolation *E. coli*

This was done at DVTD Bacteriology laboratory according to the Standard Operation Procedures in the laboratory. Samples were first enriched in buffered peptone water, inoculated on McConkey agar and incubated at 37 degrees Celsius for 24 hours. Suspected *E. coli* colonies based on colony morphology (large pink colonies) were sub-cultured on blood agar to obtain pure colonies. Biochemical tests (indole, catalase, oxidase) and Gram stain were carried out to confirm *E. coli* isolates. Gram negative, indole positive, catalase positive and oxidase negative isolates were

presumed to be in the Enterobacteriaceae family. To identify *E. coli*, the isolates were further incubated on the API 10S test strips (BioMerieux, South Africa) using the APIWEB® software.

3.3 Isolation of *Enterococcus*

*S*amples were inoculated on McConkey agar and then incubated at 35 degrees Celsius for 24 hours. Pin-point red colonies were sub cultured on Blood agar to obtain pure colonies. Grams stain, and biochemical tests (catalase, oxidase) were done on suspect isolates. Gram-positive catalase negative isolates were subjected to Streptoccal grouping using the Streptex kit from Thermo Fischer Scientific. Group D isolates were regarded as *Enterococcus* species. Suspect *Enterococcus* species were further subjected to biochemical tests (Lactose, Mannitol, Raffinose, Salacin, Sorbitol, Trehalose, Aesculin, 6.5% NaCL, Maltose, Ribose, Arabinose) with a 0.5 McFarland standard to identify the species.

3.4 Minimum Inhibitory Concentration testing

The micro dilution method was employed using the Thermo Scientific Sensititre precoated commercial plates. For *E. coli*, the Sensititre GN2F plate with antibiotic profile for Gram negative bacteria was used. The GPN3F plate with antibiotic profile for Gram positive bacteria was used for *Enterococcus*. 5 colonies were picked and were emulsified in 4ml demineralised water standard. 10µL of the suspension was transferred into 11ml of Sensititre Mueller Hinton broth to give an inoculum of 1 x 10^5 cfu/mL and the tube vortexed. 50µL of the broth suspension was then transferred into each well of the Sensititre plate. The plate was covered with the provided

adhesive seal and incubated at 34-36°C for 24 hours. The plates were read after 24 hours and MIC recorded (the lowest antimicrobial concentration inhibiting growth).

Data Analysis

The MIC interpretative standard tables (CLSI) were used to interpret the results.

Table 3.1 CLSI (2022) MIC breakpoints for Enterococcus species

Antibiotic	MIC Breakpoint		
	Susceptible	Intermediate	Resistant
Erythromycin	≤0,5	1 to 4	≥8
Quinuptistin/dalfopristin	≤1	2	≥4
Daptomycin	≤2	4	≥8
Vancomycin	≤4	8 to 16	≥32
Tetracycline	≤4	8	≥16
Ampicillin	≤8		≥16
Levofloxacin	≤2	4	≥8
Linezolid	≤2	4	≥8
Penicillin	≤8		≥16
Rifampin	≤1	2	≥4
Gatifloxacin	≤2	4	≥8
Ciprofloxacin	≤1	2	≥4

Table 3.2 CLSI (2022) MIC Breakpoints for *E. coli*

Antibiotic	MIC Breakpoint		
	Susceptible	Intermediate	Resistant
Amikacin	≤16	32	≥64
Ampicillin	≤8	16	≥32
Ampicillin/sulbactam 2:1	≤8/4	16 over 8	≥32/16
Aztreonam	≤4	8	≥16
Cefazolin	≤2	4	≥8
Cefepime	≤2		≥16
Cefotetan	≤16	32	≥64
Ceftriaxone	≤1	2	≥4
Ceftazidime	≤4	8	≥16
Cefuroxime	≤8	16	≥32
Ciprofloxacin	≤0,25	0,5	≥1
Gatifloxacin	≤2	4	≥8
Meropenem	≤1	2	≥4
Gentamycin	≤4	8	≥16
Imipenem	≤1	2	≥4
Nitrofurantoin	≤32	64	≥128
Cefoxitin	≤8	16	≥32
Piperacillin	≤8	16	32
Ticarcillin/clavulanic acid constant 2	≤16/2	32-64/2	≥128/2
Tobramycin	≤4	8	≥16
Trimethoprim/Sulfamethoxazole	≤2/38		≥4/76
Piperacillin/tazobactum	≤8/4	16	≥32/4

The percentage resistance of both *E. coli* and *Enterococcus* for each antibiotic was calculated and descriptive statistics was used to present the data.

Ethical considerations

Approval was granted by the University of Pretoria Ethics Committee (REC031-21). A section 20 approval was also granted by the Department of Agriculture, Land Reform and Rural Development. Permits were also obtained from the department of Agriculture to move samples from HHWRS biobank to DVTD Bacteriology laboratory in Pretoria.

CHAPTER 4: RESULTS

4.1 Bacterial Isolation

In total, only 14 (27%) *E. coli* isolates were obtained from the 51 faecal swabs. Of the 14 isolates obtained, 5/14(36%) were obtained from carnivore faecal swabs and 9/14 (64%) for herbivore faecal swabs.

A total of 42 (82%) *Enterococcus* isolates were obtained from the 51 faecal swabs. 22/42 (52%) of the isolates were obtained from carnivore faecal swabs and 20/42(48%) from herbivore swabs. Of the 42 isolates, 22/42(52%) were identified as *E. faecium* and 20/42 as *E. faecalis*.

4.2 Antimicrobial susceptibility testing

4.2.1 *E. coli* species

Of the 14 *E. coli* isolates, 3/14 (21%) showed resistance to at least one of the antibiotics. Of the 3 isolates, 2 were from herbivore faecal samples and 1 was from a carnivore. 2/14 (14%) of the isolates showed intermediate susceptibility to at least one of the antibiotics.

Table 4.1 Minimal inhibitory concentration (MIC) levels for *E. coli* (n=14) against selected antimicrobial drugs.

	MIC Concentration								
Antibiotic	≤0,5	≤1	≤2	≤4	≤8	≤16	≤32	≤64	≤128
Amikacin	0	0	0	0	14(100%)	0	0	0	0
Ampicillin	0	0	0	14(100%)	0	0	0	0	0
Aztreonam	0	0	0	0	14(100%)	0	0	0	0
Cefazolin	0	0	0	13(93%)	0	0	1(7%)	0	0
Cefepime	0	0	0	13(93%)	0	0	1(7%)	0	0
Cefotetan	0	0	0	0	14(100%)	0	0	0	0
Ceftriaxone	0	13(93%)	0	0	1(7%)	0	0	0	0
Ceftazidime	0	14(100%)	0	0	0	0	0	0	0
Cefuroxime	0	0	0	10(71%)	1(7%)	1(7%)	2(14%)	0	0
Ciprofloxacin	14(100%)	0	0	0	0	0	0	0	0
Gatifloxacin	0	14(100%)	0	0	0	0	0	0	0
Meropenem	0	14(100%)	0	0	0	0	0	0	0
Gentamycin	0	0	14(100%)	0	0	0	0	0	0
Imipenem	0	0	13(93%)	1(7%)	0	0	0	0	0
Nitrofurantoin	0	0	0	0	0	13(93%)	0	1(7%)	0
Cefoxitin	0	0	0	9(64%)	3(14%)	1(7%)	1(7%)	0	0
Piperacillin	0	0	0	0	0	14(100%)	0	0	0
Tobramycin	0	0	0	14(100%	0	0	0	0	0

The breakpoint is indicated by the solid line to the right of the cell. Results are presented as counts, with percentages in parentheses.

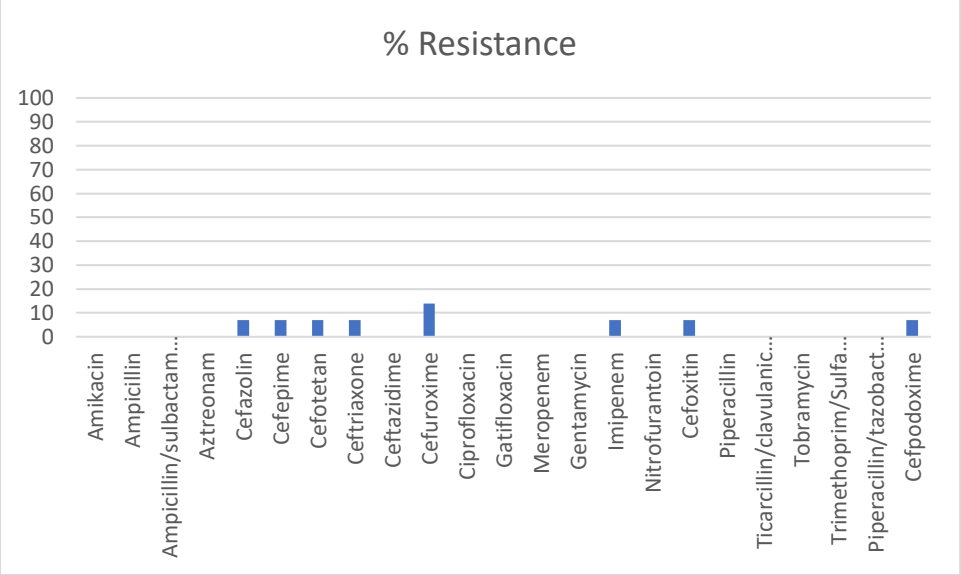

Figure 4.1 Bacterial susceptibility profile for *E. coli* isolates

Three phenotypic antimicrobial resistance patterns were observed for *E. coli* with resistance to both cefazolin-cefuroxime being observed in 1 (7%) isolate. Cefuroxime-Imipenem was observed in one isolate and a multidrug resistant isolate (cefepime-ceftriaxone-cefoxitin-cefpodoxime) was observed in one (7%) isolate.

4.2.2 *Enterococcus* species

Of the 42 *Enteroccocus* isolates, 40/42 (95%) showed resistance to at least one of the antibiotics tested. Of the 40 isolates, 22 were from carnivore swabs and 18 were from herbivore swabs. The two isolates that did not show resistance were both from herbivore faecal samples. One of the two isolates which did not show resistance to any of the antibiotics had intermediate susceptibility to erythromycin and quinupristin/dalfopristin antibiotics and the other one showed susceptibility to all antibiotics.

Table 4.2 Minimal inhibitory concentration (MIC) levels for *Enterococcus* species (n=42) against selected antimicrobial drugs.

	MIC Concentration							
Antibiotic	≤0.25	≤0.5	≤1	≤2	≤4	≤8	≤16	≤32
Erythromycin	23(55%)	1(2%)	0	7(17%)	8(19%)	3(7%)	0	0
Quinuptistin/dalfopristin	0	2(5%)	3(7%)	19(45%)	18(43%)	0	0	0
Daptomycin	0	0	4(10%)	2(5%)	6(14%)	30(71%)	0	0
Vancomycin	0	0	32(76%)	7(17%)	3(7%)	0	0	0
Tetracycline	0	0	0	41(98%)	0	0	1(2%)	0
Ampicillin	14(33%)	16(38%)	11(26%)	0	1(2%)	0	0	0
Levofloxacin	2(5%)	6(14%)	7(17%)	18(43%)	9(21%)	0	0	0
Linezolid	0	0	0	39(93%)	2(5%)	1(2%)	0	0
Penicillin	1(2%)	7(17%)	7(17%)	11(26%)	15(36%)	1(2%)	0	0
Rifampin	0	8(19%)	3(7%)	4(10%)	27(64%)	0	0	0
Gatifloxacin	0	0	34(81%)	2(5%)	6(14%)	0	0	0
Ciprofloxacin	0	10(24%)	12(29%)	13(30%)	7(17%)	0	0	0

The breakpoint is indicated by the solid line to the right of the cell. Results are presented as counts, with percentages in parentheses.

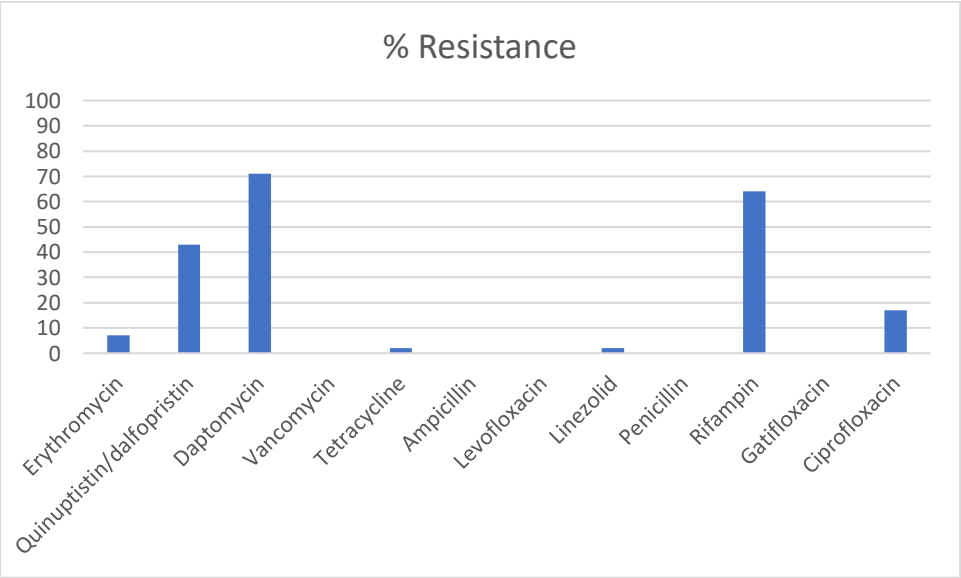

Figure 4.2 Percentage resistance of *Enterococcus* isolates to selected antibiotics

Twelve phenotypic antimicrobial resistance patterns were observed for *Enterococcus* species and the frequencies are shown in Table 4.3 below. Eighteen isolates (42%) showed multi drug resistance (resistant to 3 or more antibiotics).

Table 4.3 *Enterococcus* species antimicrobial resistance patterns

RESISTANCE PATTERN	NUMBER OF ISOLATES (n=42)	PERCENTAGE
Daptomycin	8	19
Quinuptistin/dalfopristin	4	9
Rifampin	2	5
Quinuptistin-rifampin	4	9
Daptomycin-rifampin	3	7
Quinuptistin-daptomycin-rifampin	10	24
Daptomycin-ciprofloxacin	1	2
Daptomycin-rifampin-ciprofloxacin	4	9
Erythromycin-daptomycin-rifampin-ciprofloxacin	1	2
Daptomycin-rifampin-linezolid	1	2
Erythromycin-daptomycin-tetracycline-penicillin-rifampin	1	2
Erythromycin-daptomycin-rifampin	1	2

CHAPTER 5: DISCUSSION

5.1 Isolation of Bacteria

There was a low yield of *E. coli* isolates from the faecal samples, with only 27% of the swabs yielding *E. coli* isolates. This may be due to bacterial death during the long storage time and transportation from the biobank to the lab. It may also have been due to the biobanking technique, as even freezing stool samples at -80 degrees without cryoprotectants may have reduced the number of viable bacterial cells as seen by Bilinski et al (2022). Unfortunately, the latter was not known at the time of the study. In contrast, the yield of *Enterococcus* species was much higher (82%). Based on this finding, we would recommend that cryoprotectants be used for long term storage of faecal samples for microbiological studies. While fresh samples could be used, it is not easy to transfer wildlife samples from their areas of collection to the laboratory in a quick enough time period.

5.2 MIC results of *E. coli*

Of the *E. coli* isolates, 21% were resistant to at least one of the antibiotics tested. Mupfunya et al. (2021) reported that 27% of the *E. coli* isolates from cattle in the same area were resistant to at least one antibiotic. The antibiotic profile the authors used was different to the profile used in this study, as this study relied on commercial MIC plates to allow for standardisation of results while Mupfunya et al (2021) used self-developed plates (self-developed plates was not an option at the time of this study). However, the presence of resistance in both livestock and wildlife in close proximity to each other indicates that they could be a source of resistant bacteria for each other.

Despite the low final number of cultured organisms in this study, a trend was present in that there are a number of cephalosporins which a few isolates were resistant against. Cefuroxime had the highest resistance of 14%. The other cephalosporins, namely cefazolin, cefepime, ceftriaxone, cefoxitin and cefpodoxime only demonstrated 7% of the isolate resistance. It is concerning to have a fourth generation cephalosporin (cefepime) and two third generation cephalosporins (ceftriaxone and cefpodoxime) on this list.

The WHO (2017) classified third, fourth and fifth generation antibiotics as critically important antibiotics. Nonetheless, the resistance of *E. coli* to cephalosporins in wildlife has been reported before. According to Palmeira et al. (2021), a total of 46 countries have publications reporting cephalosporin resistant bacterial isolates in wildlife. Acquired resistance in cephalosporins is mostly as a result of β-lactamases which include cephalosporinase (extended-spectrum β-lactamases (ESBL)). There is a rise in ESBL *E.coli* infections in hospitals and unfortunately third generation cephalosporins are increasingly becoming ineffective (Mughini-Gras et al. 2019).

There are very few studies on ESBL *E. coli* that have been published in the Mnisi area and for South Africa as a whole. Nzima et al (2020) reported the presence of ESBL *E. coli* in wastewater treatment plants and recipient surface water in Durban. They also discussed that this ends up contaminating the environment and rivers. In this study, with wildlife being in close proximity to a human settlement and the possibility of sewage contamination of rivers and environment, water contamination

may have played a role in the spread of resistance. Thus, there is need to investigate the presence of these resistance determinants in the river systems of the region. A review of ESBL *E. coli* in clinical human cases in this area would be a good follow up study to determine if medical use is the driver of resistance.

The other antibiotic that 7% of the isolates were resistant to was imipenem. Imipenem is a β-lactam antibiotic under the carbapenem class. Carbapenem-resistant Enterobacteriaceae (CRE) is a rising public health concern. Many studies have published the presence of CRE in companion animals, livestock and wildlife. Kock et al. (2018) did a review on studies on CRE and reported that studies done in Africa in livestock and companion animals had a prevalence of between 2 and 26%. In South Africa (North West Province), Tshitshi et al. (2020) reported that 42% of *Enteroccoccus* isolates from cattle were resistant to imipenem. More studies are required in Mnisi in livestock, human clinical cases and wildlife.

5.3 MIC results for *Enterococcus species*

In contrast to *E. coli* isolates, the resistance from *Enterococcus* isolates was marked in some of the antibiotics tested, such that 95% of the isolates were resistant to at least one antibiotic. Mupfunya et al. (2021) reported that 55% of *Enterococcus* isolates from cattle in the same area were resistant to at least one antibiotic, albeit with a different profile of antimicrobials in use. This is a cause of concern as wildlife, livestock and humans in this area can be a source of resistance genes to each other. Furthermore, 42% of isolates could be classified as multi drug resistant, which was

concerning as Mupfunya et al. (2021) did not report any multi drug resistant isolates in cattle.

The presence of multi drug resistant *Enterococci isolates* from wildlife has been published by many authors. de Araujo et al (2020) reported 66% multi drug resistant *Enterococci* isolates from wild foxes in Brazil; while Garcia et al (2022) and Dec et al (2020) reported 21.6% and 15% multidrug resistant *Enteroccoccus* isolates from wild mammals in Europe respectively. As with *E. coli*, the researchers believe that the presence of these resistance isolates is due to close proximity to human settlement and livestock. Sewage waste from humans and livestock may be contaminating the pastures and water for the wildlife. This requires the same investigation as indicated above. Also, the abuse of antimicrobial drugs in livestock is known to contribute to resistance and Mupfunya et al. (2021) reported that 99% of livestock farmers in this area do not fully understand what antibiotics are and yet they have access to them.

Resistance to daptomycin was the greatest (71%) and was a cause of concern as daptomycin is a lipopeptide antibiotic used to treat multi drug resistant *Enterococcus,* including vancomycin resistant *Enterococcus* species. While the researchers could not find any published literature on daptomycin resistance in wildlife, daptomycin resistance in *Enterococcus* species has been documented in clinical human cases (Bender et al. 2018), once again supporting our supposition of potential human to animal spread. The resistance is said to be associated with mutations of genes that encode regulatory pathways that coordinate the stress response of the bacterial cell envelope and enzymes that metabolize phospholipids (Bender et al. 2018).

Molecular tests for these genes will be valuable to not only characterise the said resistance, but to establish the relationship between resistance between human and animal strains.

The second highest resistance was against rifampin (64%). Rifampin resistance in wildlife has been published by may authors. de Araujo et al (2020) reported that 94% of *Enterococcus* isolates from wild foxes were resistant to this antibiotic, while Palmeira et al (2021) also published that 21% of isolates from wild animals in Portugal were resistant to rifampin. The finding is also not surprising as rifampin is used to treat tuberculosis, with South Africa estimated to have a prevalence of tuberculosis of 737 for every 100 000 people (TB Facts.org 2021). Rifampin resistance in both tuberculosis and *Enterococcus* is associated with mutations in the gene encoding the β subunit of RNA polymerase (Urusova et al. 2022). There is a possibility that the resistance in *Enterococcus* may be linked to tuberculosis treatment in humans. These findings are also concerning in that the resistance can also be spread from wildlife to humans through consumption of wild meat and environmental contamination.

The other antibiotic with high resistance is quinupristin-dalfopristin (43%). This antibiotic is also used to treat against vancomycin resistant *Enterococcus* species. The resistance of this antibiotic in wildlife has been published before, with Garcia et al (2022) reported that 63.2% of *Enterococcus* isolates from wild mammals from Spain were resistant to quinupristin-dalfopristin. Resistance to quinupristin-dalfopristin can be due to altered target sites, increase in efflux pumps or enzymatic

modification of the antibiotic (Hershberger 2004). These findings of resistance in antibiotics used to treat multidrug resistant bugs is concerning, as the immediate source of resistance is not known considering the expense in using the drug combination by rural communities. With the wildlife species being in close proximity to livestock and human settlement in the area, if anthropogenic activities are not the source of resistance genes, the human population could potentially be at risk of transmitting resistance genes from these animals. The possibility of intrinsic resistance cannot be ruled out at well.

The other antibiotics showed low resistance profiles. Resistance against ciprofloxacin was 17%, and much lower than the 36.5% and 40% resistance against this antibiotic in wildlife by Dec et al (2020) and de Araujo et al (2020) respectively. With ciprofloxacin being used in human medicine to treat a number of infections, there is a chance that the origin of the resistance genes may be from human resistant strains since the wildlife are in close proximity with humans. Resistance against erythromycin was only 7%, contrary to other studies which have recorded higher levels. In Brazil, de Araujo et al (2020) reported that 72.6% of isolates from wild foxes were resistant to erythromycin and 25% resistance was also reported in Italy (Dec, 2020).

Another antibiotic which had an unexpected low resistance was tetracycline (2%). Dec et al (2020) and de Araujo et al (2020) reported 36.5% and 26% resistance against tetracycline from wildlife respectively. The tetL and tetM resistance genes have been identified from isolates from wildlife (de Araujo et al, 2020). Since there is

extensive usage and abuse of tetracyclines in poultry and livestock, the researchers would have expected a much higher resistance. In Gabon, Ekore et al. (2022) reported 84% resistance in *Enteroccocus faecium* isolates from poultry, 59% for swine and no resistance from cattle. There are currently no published studies on tetracycline resistance from livestock in this area.

The results of this study together with other previous studies indicate that wildlife could be playing an important part in the epidemiology of antimicrobial resistance. More research is needed in the wildlife species to fully understand the role they play in the epidemiology of antimicrobial resistance. A One Health approach is necessary to tackle the problem of antimicrobial resistance.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

6.1 Limitations

The sample size used in this study is too small. The sampling method was by convenience and this normally results in selection bias. The sample storage was inadequate without the use of cryoprotectants.

6.2 Conclusion

In conclusion, resistance was low in *E. coli* isolates and high for *Enterococcus* isolates. The high resistance may be attributed to the close association between wildlife, livestock and human settlement which may result in dissemination of antimicrobial resistance between them. It can also be concluded that wildlife is an important determinant and source of antimicrobial resistance.

6.3 Recommendations

Based on the findings of this study, it is recommended that further studies be undertaken in the area with larger sample size, and on fresh samples. The writer also recommends more concomitant studies in human, livestock and environment (rivers, waste water, soil) to fully understand the determinants of antimicrobial resistance in the area. The findings of this study should be presented to policy makers in the area. The writers recommend periodic surveillance of AMR in wildlife, livestock and human population in the area and implementation of the AMR strategic framework in South Africa.

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agriculture, land reform
& rural development

Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform and Rural Development, Private Bag X138, Pretoria 0001
Enquiries: Ms Mamo Laing • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: MamaL@dalrrd.gov.za
Reference: 12/11/1/1/5 (2339KL)

Dr Lawrence Mugochi
University of Pretoria
Cell: 071 831 1857
E-mail: nmugochi@gmail.com

Dear Dr Mugochi

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984)

Your application dated 24 January 2022 requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. The research project is approved as per the application dated 24 January 2022 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this research project under this Section 20 permit. Please apply in writing to MamaL@dalrrd.gov.za;
3. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 permit. Please apply in writing to MamaL@dalrrd.gov.za;
4. No live animals may be used in this research project and no new samples may be collected for this research project;

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Faculty of Veterinary Science

Research Ethics Committee 08 November 2021

CONDITIONALLY APPROVAL

Ethics Reference No	**REC031-21**
Protocol Title	**Antimicrobial resistance in indicator bacteria in wildlife at a human livestock wildlife interface in Mnisi, Mpumalanga**
Principal Investigator	**Mr L Mugochi**
Supervisors	**Prof V Naidoo**

Dear Mr L Mugochi,

We are pleased to inform you that your submission has been conditionally approved by the Faculty of Veterinary Sciences Research Ethics committee, subject to other relevant approvals.

Please note the following about your ethics approval:

1. Please use your reference number (REC031-21) on any documents or correspondence with the Research Ethics Committee regarding your research.
2. Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
3. Please note that ethical approval is granted for the duration of the research as stipulated in the original application for post graduate studies (e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
4. The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

1. The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.
2. **Applications using Animals:** FVS ethics recommendation does not imply that AEC approval is granted. The application has been pre-screened and recommended for review by the AEC. Research may not proceed until AEC approval is granted.

NOTE: Conditionally approved (pending obtaining other relevant approvals).

We wish you the best with your research.

Yours sincerely

PROF M. OOSTHUIZEN
Chairperson: Research Ethics Committee

100 YEARS

Room 0-6, Arnold Theiler Building
University of Pretoria, Faculty of Veterinary Science
Private Bag X04, Onderstepoort, 0110, South Africa
Tel +27 (0)12 529 3090
Email: marie.watson-erlank@up.ac.za
www.up.ac.za

Faculty of Veterinary Science
Fakulteit Veeartsenykunde
Lefapha la Disaense tša Bongakadiruiwa

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Faculty of Veterinary Science
Animal Ethics Committee

18 May 2022

Approval Certificate
Amendment 1

AEC Reference No.: REC031-21 Line 2
Title: Antimicrobial resistance in indicator bacteria in wildlife at a human livestock wildlife interface in Mnisi, Mpumalanga
Researcher: Mr L Mugochi
Student's Supervisor: Prof V Naidoo

Dear Mr L Mugochi,

The **Amendment** as supported by documents received between 2022-04-11 and 2022-05-03 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2022-05-03.

Please note the following about your ethics approval:

1. The use of additional samples is approved:

Samples	No Approved	Additional Required	Total Approved
buffalo - fecal matter – (Stored Historic/retrospective)	1	2	3
civet - fecal matter - (Stored Historic/retrospective)	1	0	1
elephant - fecal matter - (Stored Historic/retrospective)	1	2	3
hippo - fecal matter - (Stored Historic/retrospective)	0	10	10
jackal - fecal matter - (Stored Historic/retrospective)	1	0	1
leopard - fecal matter - (Stored Historic/retrospective)	1	4	5
lion - fecal matter - (Stored Historic/retrospective)	1	3	4
pangolin - fecal matter - (Stored Historic/retrospective)	1	1	2
rhino - fecal matter - (Stored Historic/retrospective)	1	8	9
spotted hyena - fecal matter- (Stored Historic/retrospective)	1	6	7
wild dog - fecal matter - (Stored Historic/retrospective)	1	7	8

2. Please note that the approved date(s) from the original application certificate / annual renewal certificate will be applicable to this amendment.
3. Please remember to use your protocol number (REC031-21) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8454
Fax +27 12 529 8324
Email: marleze.rheeder@up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Disaense tša Bongakadinawa

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Faculty of Veterinary Science
Animal Ethics Committee

30 November 2021

Approval Certificate
New Application

AEC Reference No.:	REC031-21
Title:	Antimicrobial resistance in indicator bacteria in wildlife at a human livestock wildlife interface in Mnisi, Mpumalanga
Researcher:	Mr L Mugochi
Student's Supervisor:	Prof V Naidoo

Dear Mr L Mugochi,

The **New Application** as supported by documents received between 2021-07-12 and 2021-11-22 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2021-11-22.

Please note the following about your ethics approval:

1. The use of samples is approved:

Samples	Number
Buffalo - fecal matter (Stored- Historic/Retrospective)	1
Civet - fecal matter (Stored- Historic/Retrospective)	1
Elephant - fecal matter (Stored- Historic/Retrospective)	1
Jackal - fecal matter (Stored- Historic/Retrospective)	1
Leopard - fecal matter (Stored- Historic/Retrospective)	1
Lion - fecal matter Stored (Historic/Retrospective)	1
pangolin - fecal matter Stored (Historic/Retrospective)	1
Rhino - fecal matter Stored (Historic/Retrospective)	1
Spotted hyena - fecal matter Stored (Historic/Retrospective)	1
Wild dog - fecal matter Stored (Historic/Retrospective)	1

1. Ethics Approval is valid for 1 year and needs to be renewed annually by 2022-11-30.
2. Please remember to use your protocol number (REC031-21) on any documents or correspondence with the AEC regarding your research.
3. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
4. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
5. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8054
Fax +27 12 529 8324
Email: marleze.rheeder@up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Disaense tša Bongakadimawa

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Faculty of Veterinary Science
Animal Ethics Committee

16 May 2023

Approval Certificate
Annual Renewal
(EXT1)

AEC Reference No.: REC031-21 Line 3
Title: Antimicrobial resistance in indicator bacteria in wildlife at a human livestock wildlife interface in Mnisi, Mpumalanga
Researcher: Mr L Mugochi
Student's Supervisor: Prof V Naidoo

Dear Mr L Mugochi,

The **Annual Renewal** as supported by documents received between 2023-03-20 and 2023-05-02 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2023-05-02.

Please note the following about your ethics approval: Please note if you need to use the samples if in future a valid section 20 permit is necessary. (current permit expired in Dec 2022)

1. The use of species is approved:

Samples	Approved
buffalo - fecal matter – Stored/retrospective	3
civet - fecal matter - Stored/retrospective	1
elephant - fecal matter - Stored/retrospective	3
hippo - fecal matter - Stored/retrospective	10
jackal - fecal matter - Stored/retrospective	1
leopard - fecal matter - Stored/retrospective	5
lion - fecal matter - Stored/retrospective	4
pangolin - fecal matter - Stored/retrospective	2
rhino - fecal matter - Stored/retrospective	9
spotted hyena - fecal matter - Stored/retrospective	7
wild dog - fecal matter - Stored/retrospective	8

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2024-05-16.
3. Please remember to use your protocol number (REC031-21) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5. All incidents must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

Room 5-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8454
Fax +27 12 529 8324
Email: marleze.rheeder@up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Disaense tša Bongakadimelwa