Faculty of Sciences

Department of Biological Sciences and Biotechnology

CHARACTERIZATION OF EXTENDED-SPECTRUM-β LACTAMASES (ESBLs) AND OTHER RESISTANT GENES ENCODING BACTERIA FROM A RURAL COMMUNITY SETTLEMENT

by

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A Thesis Submitted to the Faculty of Sciences in Partial Fulfilment of the Requirements for the Award of the Degree of Master of Science in Biological Sciences of BIUST

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November 2021

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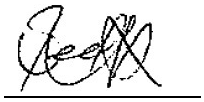
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ACKNOWLEDGEMENTS

It is with greatest pleasure to extend my sincere gratitude to my supervisor Dr Teddie O. Rahube for the continuous support throughout this study. I would like to thank him for the motivation, inspirational guidance and for having faith in me. He instilled in me the knowledge in Microbiology and molecular biology as well as trained me on how to conduct a research project independently.

My greatest gratitude also goes to my colleagues who have been giving me their utmost support and encouragement throughout my study. I am thankful to the laboratory technicians who also provided guidance and support during my experimentation in the laboratories.

I would like to thank Botswana International University of Science and Technology who has been the backbone of this project by providing financial support through the Postgraduate Research grant.

DEDICATION

I am indebted to my loving, supportive and caring mother, husband, and the guidance from God. They trusted and believed I will make it despite all the challenges that I faced. The motivational and encouraging words from them is what pulled me through each day during challenging times and to them I dedicate this work.

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ABSTRACT

Antimicrobial resistance (AMR) is regarded as a global health threat, characterized by rising resistant bacteria and rapid development of resistance genes to existing antibiotics used in clinical and veterinary settings. AMR has negative impacts and affects human, animal, and the environment. In Botswana, Africa, AMR is not well understood, particularly in rural settings with poor sanitation. The misuse and overuse of commonly used antibiotics like ß-lactam antibiotics, combined with the complex environment in poor rural communities, could contribute to the high occurrence and diversity of extended ß-lactamase (ESBL) encoding bacteria that are difficult to treat. To understand the spectrum of AMR in Botswana, it is critical to use the one health approach to characterize the occurrence and diversity of antibiotic resistance.

The study was conducted in the town of Palapye, in a rural Boseja ward, and focused on ESBLs encoding bacteria and resistance genes isolated from various water, soil, and healthy animal feces within a single household. In this study, the characterization of ESBL encoding bacteria from a rural community settlement was explored using two main approaches: the culture dependent (isolation) and culture independent (genomics) methods. In the culture dependent method, viable bacteria from animal feces and environmental samples (such as soil and pond water) were isolated using selective agar, and then randomly selected isolates were tested for phenotypic antibiotic resistance profiles on nutrient agar supplemented with six ß-lactam antibiotics (penicillin (16 g/ml), ampicillin (32 g/ml), cephalosporin (32 g/ml), meropenem (4 g/ml), cefotaxime (64 g/ml), and cefoxitin (32 g/ml)). Following that, DNA from bacterial isolates and uncultured samples were explored using a culture independent approach involving next generation sequencing (NGS) methods such as whole genome, shotgun metagenomics sequencing method and bioinformatics tools. Based on the output from NGS (whole genome sequence and shotgun metagenomics) online analysis tools were used to assemble the raw reads into consensus contigs using PATRIC (Unicycler). The contigs were further analyzed for antibiotic resistance genes (ARGs), virulence and plasmids using ResFinder, CARD/RGI, VirulenceFinder and

PlasmidFinder bioinformatics programs, respectively. Additionally, the species present in the whole genome bacterial isolate was determined by KmerFinder whereas in the shotgun sequence data, taxonomic classification was achieved by Kraken2 which revealed bacterial diversity on animal feces and environmental sources samples.

A total of 21 samples from pond water (n=3), different animal feces (such as chicken, dogs, ducks) (n=9) and different soil samples (n=9) which were collected in triplicates from a single household were cultured to isolate viable microorganisms. Overall, both selective media plates had growth for all the samples which were equally picked for further characterization. A total of 336 isolates were randomly picked from the triplicate plates from each sample source and were analyzed for antibiotic susceptibility test against six ß-lactam antibiotics, where 42.9 % (144/3360) were from different animal feces, 42.9 % (144/336), were from surrounding soil samples and 14.2 % (48/336) were from pond water. There was an overwhelming resistance to all the six ß-lactam antibiotics across all 336 isolates from animal feces, surrounding soil samples and pond exhibited 100 % resistance to penicillins (penicillin and ampicillin), 100 % resistance to cephalosporins (Cephalosporin, 2nd generation cephalosporins: Cefoxitin, 3rd generation cephalosporin: cefotaxime) and 100 % resistance to carbapenem (meropenem).

The results of NGS of cultured and uncultured samples revealed a diversity of genes encoding resistance to various antibiotics, including ß-lactam antibiotics (*bla*$_{SHV}$, *bla*$_{\text{OXA,}}$ *bla*$_{\text{TEM}}$, *bla*$_{\text{OKP-B,}}$ *bla*$_{\text{CMY}}$), tetracycline (*tet*B(P), *tet*(J), *tet* (W), *tet*(Q)), phenicol (*cat*, *cat*A3), aminoglycosides (*aph*(6)-Id, *aac*(6')-iid), macrolides (*mef*(A)), trimethoprim (*dfr*A14, *dfr*A15), fluoroquinolone (*Oqx*A, *Oqx*B) and sulfonamide (*sul* 1, *sul* 2). Furthermore, plasmid groups revealed from the samples were ColpVC, *Col*RNAI, *Col* (MG828), *Col*3M, *Col* (BS512), *Inc*R, and *Inc*FIB(K), and the virulence related genes namely colicin gene (*cia*), tellurium ion resistance gene (*ter* C), ferric aerobactin receptor gene (*iut*A), ABC transporter protein gene (*mch*F), Outer membrane protein gene (*tra*T), and glutamate decarboxylase gene (*gad*) were detected from the various samples. Chromosomal mutations were also detected (*gyr*A, *gyr*B, *par*A, *Omp*K35, *Omp*K36, *Omp*K37).

The bacterial genome sequencing revealed the cultured dog feces sample to be a mixed culture containing five bacterial organisms namely, *Proteus mirabilis* strain (CRPM10), *Citrobacter* sp. (RHB21-C05), *Paeniclostridium sordellii* strain (AM370), *Proteus mirabilis* strain (AR_0059) and *Proteus mirabilis* strain (PmSC1111) respectively. Taxonomic profiling from shotgun metagenomic analysis revealed the presence of different microbial communities in animal feces (dog) and environmental sources. The most prevalent species in animal feces, pond water and soil sample were shown to be *Klebsiella* with 44 %, 49 % and 44 % respectively. It was followed by *Proteus* species with relative abundance from animal feces (42 %), pond water and soil with 39 % and 40% accordingly. *Escherichia* has shown to be the least discovered across all samples with animal feces (4 %), pond water (4 %) and soil sample (8 %).

This study remains critical in Africa, and highlights the importance of AMR surveillance, and efforts towards the implementation of NGS to provide comprehensive information on the occurrence and diversity of AMR and mobile genetic elements from clinical and environmental sources. The research will also aid in the recommendations for community education on antibiotic use, prevention, and control measures in order to limit the spread of antibiotic resistance in community settings.

Keywords: antimicrobial resistance, rural community, antibiotic resistant bacteria, ß -lactamase, one health, next generation sequencing, whole genome sequencing, shotgun metagenomics

CHAPTER 1:INTRODUCTION

Antimicrobial resistance (AMR) is one of the biggest challenges facing global health in the current era and has resulted in the limitation towards the efficient treatment of microbial infections (Prestinaci et al., 2015) and Botswana is one of the hit countries faced with this invisible pandemic due to the increase in excessive and improper dispensing of antibiotics across different sectors (Tiroyakgosi et al., 2019). Antimicrobials are substances that target a larger range of organisms (bacteria, fungi, virus, protozoa) in general, whereas antibiotics are substances that target bacteria specifically in order to combat bacterial illnesses (Purssell, 2020). O'Neill (2016) stated that without viable antimicrobials the global economy will be immensely affected in managing AMR related diseases every year. This statement was emphasized by World Health Organization (WHO) when they predicted that by year 2050, more than ten million AMR related deaths each year worldwide will have occurred (World Health Organization, 2020) as well as driving up to twenty-four million people into severe poverty. Therefore, it is crucial for researchers to produce novel and innovative ways to combat antimicrobial resistance (O'Neill, 2016) along with updated antimicrobial utilization across different sectors of one health (Badau, 2021).

The frequent usage and abuse of antimicrobials in human and veterinary therapy has become a constant contributor to the rise of multiple antimicrobial resistance (MAR) and extensively drug resistant (XDR) bacteria. MAR bacteria are not only restricted to clinical environments but have also been discovered in non-clinical environments such as agriculture and the food industries (Aslam et al., 2018), hence the implementation of one health initiatives to track and monitor the spread and dissemination of AMR (Ewers et al., 2012). The emergence of these MAR poses a threat to all sectors of the public health which contributes to the major socio-economic and environmental problems.

There is a new superiority list of bacterial pathogens relating to health complications that the WHO has deemed of significant importance for innovation and improvement

of current and new antimicrobials (Tacconelli et al., 2018). Even though, a pursuit in discovering new novel antimicrobials is ongoing, there is disappointedly minimal discoveries over the years. The research cannot keep up with the rise of the increasing resistance that occurs around the clock. The weight and rapid dissemination of multidrug resistant organisms remain uncertain as the numbers of treatment failures continue to grow exponentially and currently the emphasis is mainly on protection from resistance to the last resort antibiotics (Borquaye et al., 2019) in order to attempt to lessen the multidrug resistance to our last hope of antimicrobials.

Bacteria act as pools of naturally occurring bacterial resistance. It means that not only does bacterial resistance occur naturally, but it has intensified with evolution because of intensive usage and environmental pollution of antibiotic residues. Antibiotic resistance has affected lower- and middle-income countries (LMICs), urban and peri urban environments as there are higher interactions among human, livestock, and immediate environment within the same community (Khan et al., 2020). Antibiotic resistance is considered as a one health challenge that has immensely affected the LMICs due to the incorrect handling of antibiotics and over the counter prescriptions for both medical and veterinary reasons. Therefore, it has been demonstrated that the rural and peri-urban communities have become a hot spot for antibiotic resistance as animals, human and environment interlinks consequently influencing cross transmission of resistance across different sources (Nadimpalli et al., 2018).

The informal keeping of livestock such as cattle, poultry, and goats within households and free-range practices in many parts of rural and even urban areas in Botswana is an indicator of the high-risk potential of antibiotic resistance transmission within the community, which influenced this study. Normally, in these communities, there are people who live in poverty and hence have poor infection prevention control due to insufficient sanitary settings (e.g., pit latrines) and lack of knowledge on good hygiene practices such as washing hands and food-handling. The conditions that define these poor communities can be a major source of exposure and thus mediating the spread of antibiotic resistance determinants (Nadimpalli et al., 2020). Furthermore, there are usually vulnerable groups of people such as children under five years, the elderly and

immunocompromised individuals living under poverty conditions in these communities, and therefore they are more at risk of greater exposure to antibiotic resistance infections (Manyi-Loh et al., 2018). The infections caused by MAR bacteria such as ESBL encoded pathogens can be exceedingly difficult to treat in overpopulated and vulnerable groups within rural and poor communities (Shaikh et al., 2015).

Additionally, other practices to alleviate poverty, such as rearing of domestic animals (dogs, cats, pigs, and poultry) in households, as well as usage of wastewater to irrigate backyard gardens (Onalenna & Rahube, 2019) also play a significant role. The information on appropriate usage and discarding of antibiotics is also lacking in rural and peri urban settlements (Cambaco et al., 2020). Therefore, it is imperative that the dissemination and transfer of ARB and ARGs within poor communities in Botswana be monitored in the interest of public health. The mobile genetic elements (MGEs) such as plasmids, bacteriophages, and transposons (Wilke et al., 2005; Rodríguez-Beltrán et al., 2020) are the main vectors of antibiotic resistance genes (ARGs) leading to rapid development and spread of MAR in bacterial communities (Mathers et al., 2015). The mobility of ARGs is high due to selection pressures from antimicrobial residues and they can be maintained and shared by bacteria of the same or different species. As a result, serious interventions and invention of new innovative antimicrobial surveillance systems are critical to track and ultimately reduce the spread of multidrug resistance among bacterial communities.

β-lactams are antimicrobials which encompasses a β-lactam ring in their molecular structure (Turner, 2005). They include penicillins, cephalosporins, monobactams and carbapenems which are frequently prescribed antibiotics to treat common bacterial infections in many health facilities worldwide including Botswana (Wilke et al., 2005 Renuart et al., 2013;Mashalla et al., 2017). More importantly, carbapenems are considered the last resort in fighting bacterial infection (Rawat & Nair, 2010). Unfortunately, most bacteria especially Enterobacterales such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus* spp. have progressively developed resistance against β-lactam antibiotics mainly penicillins class as they are widely used both in

clinical and veterinary medicine (Kimera et al., 2020). This is due to their benefits of being less toxic, effective, and easily accessible over the counter (Harris, 2015).

The emergence of extended spectrum β-lactamases (ESBLs) has been on a rise across the globe affecting prescription of β-lactam antibiotics and making common infection incurable (Shaikh et al., 2015). These enzymes are produced by various bacteria that can cleave the complex bond of the β-lactam ring rendering it ineffective in killing bacteria causing infections (Dhillon & Clark, 2012). These enzymes accelerate the increase of resistance to commonly used antibiotics such as penicillins, cephalosporins, cephamycins, and carbapenems resistance (Paterson & Bonomo, 2005). This pandemic calls for concern as fewer drugs are now available for treatment due to the rise of ESBLs especially in developing countries (World Health Organization, 2021).

1.1 Statement of the problem

The rise of ESBLs resistance is a global predicament that calls for attention as the β-lactams are increasingly considered to be ineffective for treating common and previously treatable bacterial infections such as diarrhea, urinary tract infections, and contagious diseases (Tornberg-Belanger et al., 2022). The class of β -lactam antibiotics has been widely used for the treatment of common infections in clinical settings and as prophylaxis both in human and veterinary medicine.

The highly affected communities especially in LMICs have experienced a high rise and burden of AMR (Nadimpalli et al., 2020). The spread of ESBLs in LMICs remain relatively uncharacterized albeit the frequent usage of β-lactam class of antibiotics. In Botswana, Palapye as an example, the community is faced with a serious human health threat considering the lifestyle, and socio-ecological conditions. The use of pit latrines (that have poor sanitation and open defecation), the rearing of livestock around households, storage of drinking water in unsanitary conditions all contribute to a surge in infectious disease spread in the community (Hartinger et al., 2021). In this study, the main spotlight was on the impoverished heterogeneous community of Palapye that still engages in a rural lifestyle and has limited or lack knowledge around antibiotic use and/or misuse, and the implications to human health (Mashalla et al., 2017).

These communities are characterized by vulnerable groups such as the elderly, children under the age of five who are exposed to poor living conditions in dilapidated shelters, close interactions with domesticated animal and overall poor waste management that results in contaminations of drinking water. The waste contaminated water sources such as man-made ponds used by domestic animals and for irrigation of backyard gardens in these rural communities can further serve as reservoirs for development and dissemination of AMR among animals, humans, and environment. Palapye is fast becoming an industrialized town, and because of human migration by people in search for jobs and education, diseases and AMR dynamics must be a major concern.

In Botswana, presently there is little data regarding ESBLs resistance, AMR dissemination and monitoring across these complex community settings involving human and animal interactions. Therefore, it is very crucial to understand the AMR dynamics in these developing rural and peri-urban environment and focus primarily on piloting the occurrence of ESBLs in animals and the environmental sources linked to human communities within Palapye.

1.2 Hypothesis

In this study, the hypothesis is stated that the rural community within Palapye, Boseja ward have relatively high occurrence and diverse ESBLs associated with different environmental sources (water, soil, and animal feces). The hypothesis is linked to observations of poor infrastructure, poor sanitation and high interaction of humans and domestic animals within the same community. It is also plausible to assume there is the inappropriate usage of antibiotics especially β-lactams because of lack of community health education and poor health facilities.

1.3 Objectives

To address the hypothesis, this study's main objective was to determine the occurrence of antibiotic resistance genes, including those from the ESBLs encoding bacteria isolated from animals and the environmental sources in Palapye, Boseja ward. To accomplish this objective, the following specific objectives were set.

1. To isolate ESBL producing bacterial strains from animal and environmental samples using culture methods.
2. To identify bacteria and classify antibiotic resistance determinants from cultured and uncultured samples using next generation sequencing methods.
3. To use bioinformatics tools to further identify mobile genetic elements and virulence genes within genomic and metagenomic DNA sequences.

1.4 Significance of study

This study is critical as a baseline to uncover the presence of ESBLs and other antibiotic resistance patterns in a potentially high-risk community setting. Data from this study will inform future research on the state of antibiotic resistance and recent ESBLs dynamics resulting in possible replication and expansion of the study to include other communities directly linked to animals in other rural communities around Botswana. The Government health departments will also benefit from knowledge generated and use the information to sensitize the community on the potential routes of antibiotic resistance spread and how they can protect themselves. Awareness on the usage and misuse of the antibiotics which may contributes to the rise in antibiotic resistance and mortalities in the community will also be raised. The study has the potential to inform the policy makers and health care providers (e.g., Ministry of Health and Wellness) on the execution of antibiotic prescription plans across rural community settings and make appropriate dispensation of the latest and critically important ß-lactam (e.g., carbapenems) antibiotics to minimize resistance development. Moreover, this information will also be important for both local and global (WHO) AMR surveillance programs that can be used to guide efforts on antibiotic stewardships including National Action Plan (NAP) in relation to antibiotic resistance in Botswana.

CHAPTER 2:LITERATURE REVIEW

2.1 Overview of β-lactam resistance

Since Alexander Fleming discovered the first β-lactam antibiotic, penicillin, ninety-three years ago, β-lactam antibiotics have remained one of the most extensively used antibiotics in the world (Fleming, 1980; Gaynes, 2017). As a result of this discovery, resistance to most bacterial infections caused by both Gram-negative and Gram-positive bacteria has risen at an exponential rate (Lobanovska & Pilla, 2017). Antibiotic development has also reached a major 'milestone,' with millions of antibiotics being produced and used, but resistance has grown, eroding researcher's efforts and hard work in keeping up with finding new and novel antibiotics consequently leading to an increase in antibiotic resistance related deaths globally (Kong et al., 2010).

Challenged with the reality of high mortalities, it is crucial to analyze antibiotic resistance development globally in different sectors (e.g., clinical, agricultural, and environmental) by merging different interventions and fast analytical methods in order to prevent the escalating global crisis (Founou et al., 2016). Common laboratory techniques continue to be used, such as microbiological culture, molecular biology, antibiotic susceptibility tests to identify and analyze the impacts of AMR. Furthermore, new diagnostic methods are also being developed to provide surveillance information that can help in public health systems infection control and track the spread of resistance to critical antibiotics which include the last resort class.

The well-known β-lactam include penicillins, cephalosporins that are categorized by generations (first, second, third, and fourth generation cephalosporins), carbapenems and monobactams (Page, 2011). Most of the examples of these commonly used β-lactam antibiotics in clinical and veterinary settings are Penicillins (such as ampicillin, amoxicillin), Cephalosporins (such as ceftazidime and cefotaxime), Carbapenems (such as meropenem imipenem) and Monobactams (aztreonam) (Table 2.1.1).

Table 2.1.1: β-lactam antibiotics groups and their spectrum of action

Antimicrobial group	Examples	Spectrum of action
Penicillins		
a) Natural Pencillins	Penicillin G (injection) Penicillin V (oral)	Primarily effective against Gram-positive bacteria
b) Penicillinase	Cloxacillin, dicloxacillin, methicillin, nafcillin, oxacillin	Effective on infections caused by β-lactamase–producing staphylococci
c) Aminopenicillins	Amoxicillin, ampicillin, amoxicillin/clavulanate, ampicillin/sulbactam	Effective against both Gram-positive and Gram-negative bacteria
d) Carboxypenicillins	Carbenicillin, ticarcillin, ticracillin/clavulante	Greater effect against Gram- negative bacteria
Cephalosporins		
a) 1st generation	Cefadroxil, cefazolin, cephalexin, cephalothin, cephradine	Effective against Gram-positive bacteria and limited Gram-negative bacteria.
b) 2nd generation	Cefaclor, cefamandole, cefmetazole, cefoxitin, cefoprzil, cefuroxime	Most effective against some Gram- negative bacteria and Gram- positive bacteria are less affected.
c) 3rd generation	Cefixime, cefoprazone, cefotaxime, ceftazidime, ceftriaxone	Efficient on *Pseudomonas* and *Enterobacter* spp. Commonly used in the treatment of nosocomial infections
d) 4th generation	Cefepime	Highly active against Gram- positive and Gram-negative organisms, including *P. aeruginosa*
Carbapenems		
a) Carbapenems	Imipenem, Meropenem, Doripenem, Ertapenem	Active on a wide spectrum of both Gram-positive and Gram- negative microorganisms
Monobactams		
a) Monobactam	Aztreonam	Active only on aerobic Gram-negative bacteria (e.g., *Neisseria, Pseudomonas)*

Adapted from (Scholar & Pratt, 2000; Pandey & Cascella, 2021)

The mechanism of action for all β-lactam antibiotics includes the inhibition of bacterial cell wall formation through the alteration of the penicillin binding proteins (PBPs) that are responsible for the peptidoglycan synthesis in the cell wall. This action leads to a fragile cell wall leading to bacterial cell death (King et al., 2014). Penicillins and cephalosporins work in a similar way by attaching to the β -lactam ring to DD-transpeptidase enzyme on the PBPs, which is responsible for peptidoglycan cross linking activities that maintains the rigidity of the cell wall (Kong et al., 2010; Munita

& Arias, 2016). Carbapenem antibiotics work by inhibiting the peptidase domain of PBPs, which prevents the peptide cross-linking processes that occur during bacterial cell wall formation (Papp-Wallace et al., 2011). The monobactam covalently binds to PBP_3 to prevent growth of the cell wall through peptidoglycan synthesis (Barie et al., 2008).

Since the β-lactam antibiotics have been commonly used to fight bacterial infections, bacteria have developed their own mode of action against the β-lactams. This includes the alteration of the outer membrane proteins thus preventing the drug to reach its target which typically happens through porins that occurs mostly in Gram-negative bacteria (Fernández & Hancock, 2012). Another resistance mechanism occurs through alteration of the structure of penicillin binding proteins to reduce affinity to the binding site for β-lactams but not leading to bactericidal activity (Soares et al., 2012; Munita & Arias, 2016). This mechanism was observed to affect methicillin and penicillin antibiotics in Gram-positive organisms (Peacock & Paterson, 2015). The most common resistance to the β -lactam antibiotics is the production of β-lactamases enzymes which hydrolyses the β -lactam ring making the drug structure unstable (Bonomo, 2016). This mechanism of resistance has rendered almost all β -lactam antibiotics ineffective through this enzyme. They are normally chromosomally mediated but recently they have been carried in plasmids in Gram-negative bacteria, accelerating the spread among the bacterial community (Turner, 2005) .

2.1.1 Classification of Extended Spectrum β- lactamases

The β-lactamase enzymes classification is based on two systems, mainly focused on the molecular and functional structure that was elaborated by Ambler molecular system (Hall & Barlow, 2005) and the latest updated Bush–Jacoby–Medeiros functional system (Bush & Jacoby, 2010). The classification by Ambler includes class A, C, and D enzymes which hydrolyse substrates that entails an active serine site, whereas there is class B known as metalloenzymes that hydrolyse β-lactam antibiotics with the help of divalent zinc ions joined at the active site (Paterson & Bonomo, 2005;

Brink et al., 2007; Kong et al., 2010). The molecular characterization of Ambler emphasizes more on the affinity of the protein of the beta lactamase enzyme. From the latest update by Bush–Jacoby–Medeiros in 2010, the classification was centered on the functional properties of the enzymes and their capability to destroy the β-lactam antibiotics (Bush & Jacoby, 2010). The grouping of β-lactamase enzymes by Bush–Jacoby–Medeiros is demonstrating that there is group 1 (class C) which entails of cephalosporins; group 2 (classes A and D) includes the broad spectrum, inhibitor-resistant, extended-spectrum beta lactamases, serine carbapenemases and lastly group 3 (class B) which has metallo- β-lactamases.

Previously Giske et al. (2008) has added a simplified classification of how ESBLs can be characterized by dividing them in three main groups which includes $ESBL_A$ (TEM, SHV and CTX-M), which are typically inhibited by clavulanic acid, $ESBL_M$ (Miscellaneous group which comprises of enzymes encoded in plasmids [AmpC]), a group which is inhibited by cloxacillin, and finally the $ESBL_{CARB}$ which are enzymes that destroys all ß-lactam agents, as well as giving rise to carbapenem resistance. This group is hindered by Ethylenediaminetetraacetic acid (EDTA).

Although numerous ESBL families have been explained and characterized, but the prevalent ones that bestow antibiotic resistance belong to the largest group 2, representing class A and D. This group include ESBL families of TEM, SHV, CTX-M from class A and OXA from class D (Turner, 2005; Villegas et al., 2008) and all the variants of ESBL enzymes originate from group 2 which carry the most clinically significant enzymes.

2.1.2 Global β-lactam resistance surveillance

The WHO has introduced the Global Antimicrobial Resistance Surveillance System (GLASS) in 2015 to fight AMR. This action plan has included ß-lactam antibiotics (penicillin, third and fourth generation cephalosporin and carbapenems) and other drugs as the most crucial classes to test susceptibility to most bacteria found clinical

settings, including extended-spectrum ß-lactamase (ESBL) encoding bacteria (World Health Organization, 2015; Founou et al., 2019). Retrospectively, there has been a surge in the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA), extended-spectrum β-lactamase-producing (ESBL) *Enterobacterales*, carbapenemase-producing *Enterobacterales* (CRE), multi-resistant *Pseudomonas aeruginosa*, vancomycin-resistant *enterococci*, and multi-drug resistant *Acinetobacter baumannii* continue to be a threat globally (Khawaja et al., 2017).

AMR surveillance network in Europe (European Centre for Disease Prevention and Control, 2015) and Grundmann et al. (2017) also demonstrated that majority (77 %) of European countries have been affected by ESBL encoding *E. coli*, carbapenemase producing *K. pneumoniae* and *Enterobacterales* resulting in resistance to the last resort antibiotics such as carbapenems and colistin. The findings suggested the need to explore AMR determinants in all sectors of one health, not specifically on clinical isolates alone (Berendonk et al., 2015). Additionally, European regulators made it compulsory for AMR determinants to be monitored in animal and vegetable produce during food testing (European Food Safety and Authority & European Centre for Disease Prevention and Control, 2021). The food analysis was mostly on Gram-negative bacteria including ESBLs and carbapenemase encoding *Salmonella* and *E. coli* linked to food borne pathogens.

In Brazil (Jaskulski et al., 2013; Núñez-Samudio et al., 2021) and India (Shahid et al., 2011; Kayastha et al., 2020 studies indicated clinical isolates to have a combination of ESBLs genes making it difficult-to-treat infections as they have multiple ESBLs genes. Therefore, extensive emphasis should be on research based on determining the ß- lactam resistance hotspots in order to find ways to intervene with the dissemination processes as well as finding ways to interrupt the mechanism of transfer. In Thailand, a study indicated that an increase of livestock farming has become a breeding ground for multi-drug resistant bacteria, samples from various sources such as humans, pigs, chickens, and water were collected from these farms and results showed an extremely high intensity prevalence of ESBL producing *E. coli* (Boonyasiri et al., 2014; Trongjit et al., 2016). The prevalence of ESBLs encoding bacteria in hospital acquired

infections contributes to high mortality rates especially in poor communities (Lim et al., 2015). Similarly, a study in Bangladesh identified ESBL encoding *E. coli* and Carbapenem-resistant *E. coli linked* to high dissemination of ESBL resistance genes across different sectors in LMICs (Schmithausen et al., 2018).

In Africa, very few studies have described and characterized ESBL producing bacteria. Sub-Saharan Africa has lagged behind in terms of AMR surveillance and accurate localized AMR patterns to demonstrate the true extent and the bearings of AMR (Osei- Sekyere, 2016). Most African countries are still developing, and AMR continues to drive the LMICs in Africa into poverty due to socio-economic and ecological factors, and therefore the spread and dissemination of AMR especially ESBLs, remains (Chokshi et al., 2019). Previous research in Zimbabwe has documented an exceptionally high prevalence of ESBL producing bacteria (e.g., *Enterobacterales*). The high prevalence of ß-lactam resistance was reported as no surprise because these classes of antibiotics are commonly used in Zimbabwe (Mhondoro et al., 2019). The intensity of resistance to the last-resort antibiotics, including carbapenems, has also been observed in Ethiopia, Uganda, Kenya, and Burkina Faso (Kiiru et al., 2012; Kateregga et al., 2015; Moga-Siraj et al., 2015; Ouedraogo et al., 2016; Selassie, 2016; Legese et al., 2017; Kagia et al., 2019). These studies have observed that most infections caused by *E. coli* and *K. pneumoniae* have developed resistance to most antibiotics, including ß-lactams, specifically third generation cephalosporins, thus leading to a greater prevalence of ESBL producing bacteria in the hospital.

In Nigeria, a high prevalence of carbapenemase producing *E. coli* and *K. pneumoniae* has also been reported (Ogbolu & Webber, 2014; Olalekan et al., 2020). These studies rendered cephalosporins and carbapenems ineffective in the majority of Gram-negative isolates, and they also highlighted the importance of the need for constant surveillance and update of recent on AMR occurrences. Still, in Nigeria, ESBL genes were discovered in farm workers, farm environments, and feces matter from farm animals (Aworh et al., 2020).

2.2 Genomics approaches in AMR surveillance

In addition to the conventional methods that detect phenotypic AMR, studies are increasingly using next generation sequencing (NGS) methods that further characterize the AMR origin, mechanisms, and genetic determinants (Tshibangu-Kabamba et al., 2020). To combat AMR, sequencing technologies are constantly evolving, and there are now a variety of NGS platforms available, which are also becoming more affordable for use in LMICs.

Genomics approaches involve high-throughput technology that analyses genomes to produce biological data from microbial samples, either from culture or directly from uncultured samples. These techniques involving high throughput NGS have proven to be able to provide quick results and detailed taxonomy classification of pathogenic bacteria, antimicrobial resistance, plasmids, and virulence genes. In the public health sector, the techniques have shown their contributions to the growth of treatments for clinical and veterinary welfare against antibiotic resistance as well as exploration of the entire human pathogen genome and its mechanisms (Waddington et al., 2022). The main genomics approaches to AMR surveillance include whole genome sequencing (WGS) and metagenomics.

2.2.1 Bacterial whole genome sequencing

Bacterial WGS is a culture dependent technique that involves the sequencing and analyses of the entire bacterial genome including extra chromosomal elements such as plasmids, exploring its identity, genotype, antibiotic resistance, and lineage (Morganti et al., 2019). This approach has been carried out in several studies and demonstrated to be excessively beneficial in the predictions of antimicrobial resistance genes from a wide range of microorganisms isolated from different clinical and non-clinical environments.

A study carried by McDermott et al. (2016) which assessed the difference between AMR phenotypic methods and WGS methods in *Salmonella* species, revealed that the two methods complement each other, and demonstrated that WGS can be used in the identification of known antimicrobial resistance determinants as well as unknown novel resistance pathways. A study carried by Stoesser et al. (2013) also demonstrated the ability of using WGS in identifying AMR in *E. coli* and *K. pneumoniae* species and discovered variety of acquired antibiotic resistance genes and chromosomal mutation genes. A similar study was done on *Staphylococcus aureus* (Gordon et al., 2014). These studies depicted the importance of WGS in surveillance of AMR, and its ability to identify and distinguish chromosomally and plasmid mediated resistance genes (Boolchandani et al., 2019).

Therefore, the use of WGS has the potential to trace and track the lineages, evolution, and dissemination of MDR bacteria as well as other resistant pathogens found in clinical and environmental settings. Another study on *K. pneumoniae* clinical isolates was characterized using comparative genomics tools to identify AMR genes, including ß-lactamases (Kumar et al., 2011). For more examples of genomics studies in the Democratic Republic of Congo, South Africa, and Nigeria, WGS and bioinformatics tools were used in the surveillance and discovery of the prevalent antimicrobial resistance genes and resistance plasmids from clinical, animal, and environmental isolates (Jesumirhewe et al., 2017; Founou et al., 2019; Irenge et al., 2019; Aworh et al., 2020). With reference to ESBLs, a study conducted in Denmark (Hasman et al., 2015) also used WGS and bioinformatics tools to monitor and characterize ESBLs and *Amp*C encoding genes in *E. coli* from animal products, food, and human isolates.

The WGS studies further prove the efficiency in identifying various genotypic, metabolic and resistance profiles in one single analysis and improvement on microbiological data analysis of bacteria isolates (Didelot et al., 2012; Hasman, et al., 2014). With reference to most recent genomics studies, WGS technology might be the new best method in characterizing multidrug resistance in bacteria as it is fast and generates lots of data compared to conventional methods (Oniciuc et al., 2018; Baker et al., 2018; Babiker et al., 2019; Painset et al., 2020).

2.2.2 Metagenomics

Metagenomics is another genomics approach based on the analysis of community DNA recovered directly from uncultured environmental samples (Handelsman, 2005; Rashid & Stingl, 2015; Nowrotek, et al., 2019). This culture independent method is a powerful tool as it is not biased towards the favorable conditions of cultured organisms but a representation of the entire microbial community directly in a sample (Forbes et al., 2017). The metagenomics approach for bacterial characterization can be divided into two different methods, namely, the 16S rRNA gene targeted amplicon sequencing and the shotgun metagenomics "all microbes" sequencing. The targeted amplicon sequencing method is based on targeting a specific group of microbes using specific phylogenetic marker genes (e.g., 16S rRNA) for bacterial profiling (D'Amore et al., 2016). This approach is quick and affordable and gives information on the composition, organization, and variety of the microbial community (Muhamad Rizal et al., 2020), and it is based on curated reference databases.

Documented studies have demonstrated and described how both metagenomic technology approaches can be used to track and trace ARGs and mobile genetic elements from bacterial isolates and environmental samples. This enlightens and contributes to public health epidemiological surveillance, the development of comprehensive AMR determinants, and infection control (Stoesser et al., 2013; Köser et al., 2014; Zankari et al., 2017). Several studies have explored the convenience of 16S rRNA gene sequencing in the identification of species, which further informed the relatedness of the predicted microbial community to various AMR determinants (Gatica et al., 2015). Studies in China and Nigeria used a 16S rRNA gene sequencing approach in the identification of bacterial communities that were encoded with clinically relevant ARGs found in water bodies and discovered the possibility of these identified organisms being carriers of ARGs as well as drivers of the spread of resistant genes found in the water (Wang et al., 2018; Adelowo et al., 2020). In clinical isolates, 16S rRNA sequencing has been used to identify pathogenic and clinically relevant ARG encoded microorganisms (Srinivasan et al., 2015). A study by Li et al. (2019) has further shown the efficiency of using 16S rRNA sequencing in identifying the

bacterial community from different samples and further indicated that there might be a link between the ARGs detected and the predicted bacterial hosts.

The downfall of using the 16S rRNA gene approach has its limitations regarding its accuracy in determining prokaryotic taxonomic composition as it tends to omit subspecies and specific genetic data by targeting the specific gene of interest (16S rRNA) and it does not predict ARGs (Gohl et al., 2016). Another drawback to using 16S rRNA sequencing is that the primers may amplify in a non-specific region, leading to a misleading representation of the actual composition of the sample (dos Santos et al., 2019) and producing a limited amount of data for that sample (Cariou et al., 2018). Consequently, this influenced the need to further produce newer approaches such as shotgun metagenomics, which can curb some of the drawbacks provided by 16S rRNA gene sequencing (Poretsky et al., 2014; Breitwieser et al., 2017; Boers et al., 2019; Muhamad Rizal et al., 2020).

Shotgun metagenomics sequencing characterizes all microbes in a given samples by cutting the DNA in short fragments that will then further be assembled into longer contigs (consensus) and annotated to reproduce the DNA of the sample (Quince et al., 2017). Shotgun metagenomics when compared to 16S rRNA, it produces a vast amount of data that is required to produce a sufficient coverage of the sample therefore widening the scope of describing and comparing microbial community in the sample as well as predicting known and unknown variants (such as ARGs, MGEs and virulence factors) within the sample (Bharti & Grimm, 2019). This method determines the thorough genetic composition of an organisms to the strain level (Truong et al., 2017). It further provides information on the functional composition of the organism which includes metabolic, virulence and antimicrobial resistance abilities of the organism and other unknown microbial functional capabilities (Rausch et al., 2019) while 16S rRNA only targets sample microbial diversity. Additionally, it provides comprehensive data that can be compared through comparative analysis (Lapidus & Korobeynikov, 2021).

The shotgun metagenomics approach has been effectively used in numerous studies revealing known and novel antimicrobial resistance genes, including but not limited to β-lactams, tetracycline, and aminoglycosides and ancestral lineages (Allen et al., 2008; Schmieder & Edwards, 2012). The contribution of the metagenomics approach has further explained the taxonomy and origin of antibiotic resistance genes and complements data from other genomics approaches such as culturomics (high-throughput sequencing and functional analysis of viable bacterial isolates) (Bilen et al., 2018; Nowrotek, et al., 2019). Metagenomics have also been reviewed to have the capacity to detect and identify AMR in food borne pathogens across the food chain and food producing animals, therefore the public health laboratories across the world has since adopted metagenomic approaches in their laboratory analysis and tracking sources of bacterial outbreaks (Breitwieser et al., 2017; Nadon et al., 2017; Oniciuc et al., 2018; Gerner-Smidt et al., 2019; Desdouits et al., 2020; Mohammed & Thapa, 2020; NIHR Global Health Research Unit on Genomic Surveillance of AMR, 2020; Uelze et al., 2020). Mohammed and Thapa, (2020) demonstrated that with the help of metagenomic methods, the source of occurrences of AMR and dissemination among humans, animals, and the environment can be deduced and traced back to their origin.

Other studies have used metagenomics approaches to trace and investigate ARGs from different types of samples, including clinical, environmental, and animal isolates (Fitzpatrick & Walsh, 2016; Bengtsson-Palme, 2018; Khan et al., 2020; Lira et al., 2020). ARGs were recently discovered in water bodies containing various pathogenic microorganisms, including wastewater treatment plants and effluent water from clinical, industrial, and agricultural waste flows (Onalenna & Rahube, 2022). The metagenomics technique revealed more multidrug resistance genes and mechanisms, as well as the likelihood of resistance spreading via vertical and horizontal gene transfer amongst different microorganism communities because of selection pressures in polluted sources. Furthermore, the competence and throughput of the metagenomics approach have informed various ARGs exchange and the significant risk of multidrug resistant and untreatable pathogens, both of which are of public concern (Noyes et al., 2016; Guo et al., 2017; Chen et al., 2018; Das et al., 2020; Lal Gupta et al., 2020).

The metagenomics sequencing approach continues to demonstrate its capability in rapidly and efficiently processing a large number of sewage samples, detecting and quantifying microbes and AMR determinants representative of human populations (Hendriksen et al., 2019; Nieuwenhuijse et al., 2021; NIHR Global Health Research Unit on Genomic Surveillance of AMR, 2020). In China, researchers explored metagenomic methods in wastewater treatment plants and determined numerous ESBL encoding bacteria and plasmids encoding resistance to a wide variety of clinically relevant antibiotics (Jiang et al., 2019). Similarly, a study by Ajayi et al. (2021) revealed resistance associated with ß-lactam genes carried in conjugative plasmids using NGS and comparative genomics methods.

Considering the intensity and challenges attributed to AMR, more holistic approaches such as one health together with NGS methods need to be deployed. NGS technology plays a vital role in genetic analysis across all multidisciplinary principles relating to one health by producing huge amounts of data promptly as compared to traditional methods. It is therefore important to utilize this method, especially in LMICs where it is underutilized.

2.3 Bioinformatics tools in characterization of ARGs

Genomics provides huge datasets, especially from NGS, leading to larger amounts of data that require powerful computational tools to analyze. Bioinformatics is a relatively new discipline in science which associates fields of biology, computer science, and information technology and combines them together to produce biological solutions to challenges faced by the scientific community. The bioinformatics pipeline uses databases, algorithms, and machine learning intelligence to process sequence data and translate them into readable and useful biological information. The bioinformatics analyses include but are not limited to assembly, physical mapping, gene annotations, and sequence alignments for phylogenetic tree construction.

Bioinformatics is crucial for the analysis of sequence data for AMR characterization. There are several web-based, computational tools, software, and databases available for storing and analyzing NGS data. The most popular sequence free storage databases are the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov), European Bioinformatics Institute (EBI) (https://www.ebi.ac.uk) where you will find analysis tools such as Basic Local Alignment Search Tool (BLAST), open reading frame finder (ORF Finder), annotation platform, assembly archive, clustal omega, just to name a few, that allow users to visualize and assess the extracted molecular biological data from sequences. These tools have allowed the user to explore using a single query across all different available databases. Other available specialized and user-friendly AMR analysis platform examples include Comprehensive Antibiotic Resistance Database (CARD) (https://card.mcmaster.ca), Antibiotic Resistance Genes Database (ARDB) (https://ardb.cbcb.umd.edu), Centre for Genomic Epidemiology (CGE (http://www.genomicepidemiology.org), Galaxy (https://galaxyproject.github.io), Pathosystems Resource Integration Center (PATRIC) (https://patricbrc.org), Beta-Lactamase DataBase (BLDB) (http://bldb.eu) and Antibiotic Resistance Gene-ANNOTation (ARG-ANNOT) (Gupta et al., 2013). The two most common web-based bioinformatics tools used for ARGs detection and analysis are (ResFinder) from the Centre for Genomic Epidemiology (Bortolaia et al., 2020) and a combination of BLAST and the Resistance Gene Identifier from the Comprehensive Antibiotic Resistance Database (CARD).

ResFinder 4.0 is an online-based tool for identifying horizontally acquired antimicrobial resistance genes from raw reads or assembled contigs from bacterial isolates of different sources (food, animal, and human) (Clausen et al., 2016). It was first established in 2012 as one of the first user friendly platforms for researchers with no bioinformatics skills (Zankari, 2014). This tool identifies acquired ARGs using BLAST *in silico* predictions, and new resistance genes are added as they are discovered (Feldgarden et al., 2019). The ResFinder tool has been reviewed to have an advantage over other databases in having the ability to process NGS raw reads as well as assembled contigs. However, it can only give the output of acquired resistance

genes and chromosomal mutation genes are limited to a few pathogenic microorganisms (Lal Gupta et al., 2020). The ResFinder tool has close to two thousand antimicrobial resistance genes, which include ß-lactamase genes (Zankari et al., 2012).

The CARD platform runs concurrently with Resistance Gene Identifier (RGI) software, which produces the resistance genes in the database, spotting functional homologues protein model associated with antimicrobial resistance genes. CARD platform accepts data from sequenced DNA and proteins to identify AR genes (McArthur et al., 2013). Moreover, the CARD database delivers a wide spectrum of antibiotic resistance ontology (ARO) and recent ARGs data is updated. The drawback on the CARD platform is that the input data sequence DNA/protein has a size limit of 20 MB, but it has a wide range of chromosomal mutation genes that can be detected (Khezri et al., 2020)**.** The CARD platform has over one thousand curated antibiotic resistance genes in the database, including a variety of ß-lactam resistance genes as well as multidrug resistance genes (McArthur et al., 2013). ResFinder and CARD/RGI tools are therefore mostly used due to their ability to produce results in a quick time, and they are reachable tools as they are web-based tools and do not require high computer expertise to be used. The drawbacks may be that web-based tools can occasionally be defective and contain errors with broken links.

Another option used particularly by experienced bioinformatics users and scientists for sequence data analysis is through the command line interface (CLI). CLI tools are available in an open-source platform in GitHub desktop (https://github.com/ **)** where various command lines are used through the installation of a local software, and they include but not limited to Antimicrobial Resistance Identification By Assembly (ARIBA), Antibiotic Resistance gene-ANNOTation (ARG-ANNOT) and MEGARes.

The ARIBA tool is a bioinformatics pipeline which can be accessible through downloading the local installation from GitHub platforms (https://github.com/sanger-pathogens/ariba), and it uses a command line for aligning and assembling reads locally to predict AMR and sequencing typing. This software determines AMR related genes and single nucleotide polymorphisms (SNPs) directly from sequence data and short

reads to produce a thorough and comprehensible output (Hunt et al., 2017). The ARIBA tool has been identified as a powerful tool when compared to other bioinformatics tools as it uses raw sequence data to predict AMR determinants and variants related to AMR that are identified in the sequence data. The downside to this pipeline is that the users need to have expertise in executing computer commands. Moreover, the ARG-ANNOT bioinformatics tool analyses sequences without using a web-based platform (Gupta, et al., 2013). It is used to determine ARGs from bacterial organismal genomes with the aid of the BLAST algorithm in a local installed program that was last updated in the year 2018.

Another recently updated bioinformatics tool is the MEGARes tool, which comes with a high throughput antimicrobial resistance database and accurate annotation information for short reads data from metagenomic sequence analysis as well as hierarchical classification of sequences from bigger data (Doster et al., 2020). It can be accessed as a standalone software tool and recently, the AmrPlusPlus interface platform accessible through the Galaxy platform was added to aid the classification, identification, and quantification of AMR genes easier and quicker in metagenomic sequence data (https://megares.meglab.org). Similarly, DeepARG is also a tool that predicts ARGs and their determinants in metagenomic sequence data. The software has been shown to be perfectly accurate in its predictions and most advantageous since it can be accessed both through a command line and a graphical user interface to predict ARGs (Arango-Argoty et al., 2018).

Furthermore, genomics and bioinformatics capacity in Africa remains limited, and therefore it is most relevant to conduct this research as part of bridging this knowledge gap in Botswana, further promoting the use of advanced technologies such as NGS for surveillance of AMR including ß-lactam resistance. The use of genomics and bioinformatics analysis would greatly complement traditional microbiology methods and reveal more information on the occurrence and diversity of ß-lactam resistance, other ARGs and MGEs.

CHAPTER 3:MATERIALS AND METHODS

3.1 Study site

The study area is Palapye, which is one of the rapidly growing peri urban areas with an increasing population (approximately 6,211 people) (Statistics Botswana, 2011), influenced by new developments such as the establishment of academic institutions (e.g., Botswana International University of Science and Technology), the expansion of mining industries (e.g., Morupule Coal Power Station), the construction of shopping malls, and various links to tourist destinations in and around Palapye. However, other parts of Palapye remain rural despite these developments, and therefore, as a peri-urban area, Palapye is also characterized by inter-linkages of the ecosystems that are highly influenced by human and animal activities. The anthropogenic activities in Palapye include traditional agriculture, rearing livestock, and growing food.

Boseja ward 22º 33’42.05” S 027º 08’ 21.62” E is a chosen study site, located 10.8 km from Botswana International University of Science and Technology, 27 km from the municipal wastewater treatment facility and 14 km from the newly constructed shopping malls. The community lifestyle in Boseja ward is more rural, associated with poverty, poor literacy, and poor facilities. The activities in this community include backyard gardening, rearing of small livestock and usage of pit latrine toilets (including open defecation) in the same yard (Figure 3.1.A below). The socio-demographic characteristics of people in this community also include the elderly and children under five (5) years living mostly in overpopulated and dilapidated environments. The study site is representative and ideal for a one health research approach, which demonstrates the interactions of human, animals, and their environment (Figure 3.1.B).

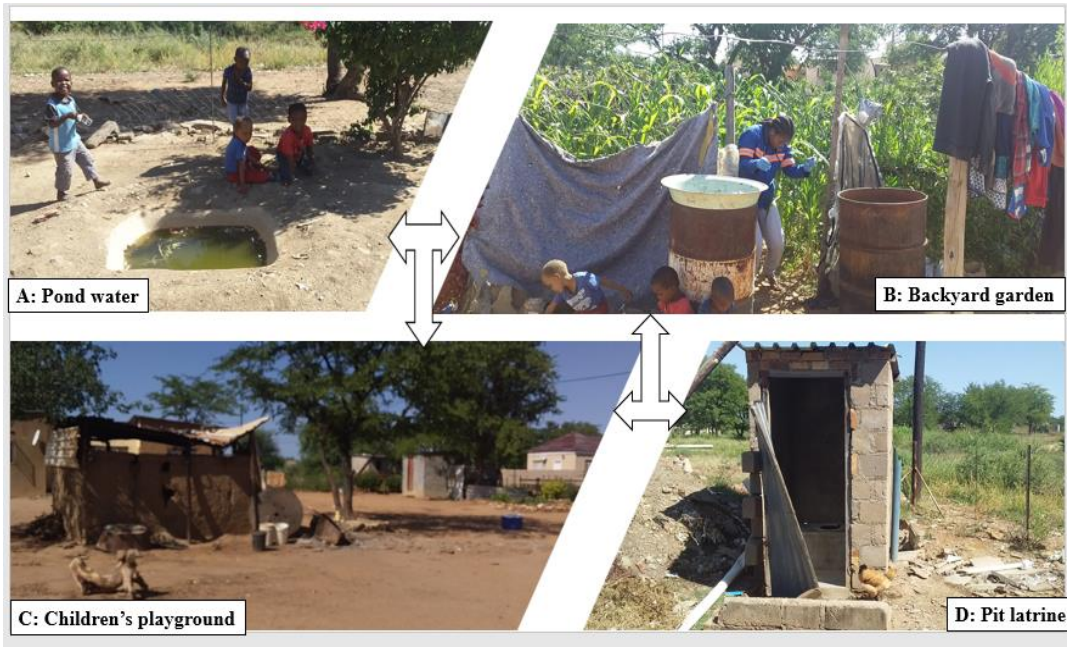


Figure 3.1.A: The compound dynamics showing characteristics of poor living conditions at the study sites (©KBrooks).

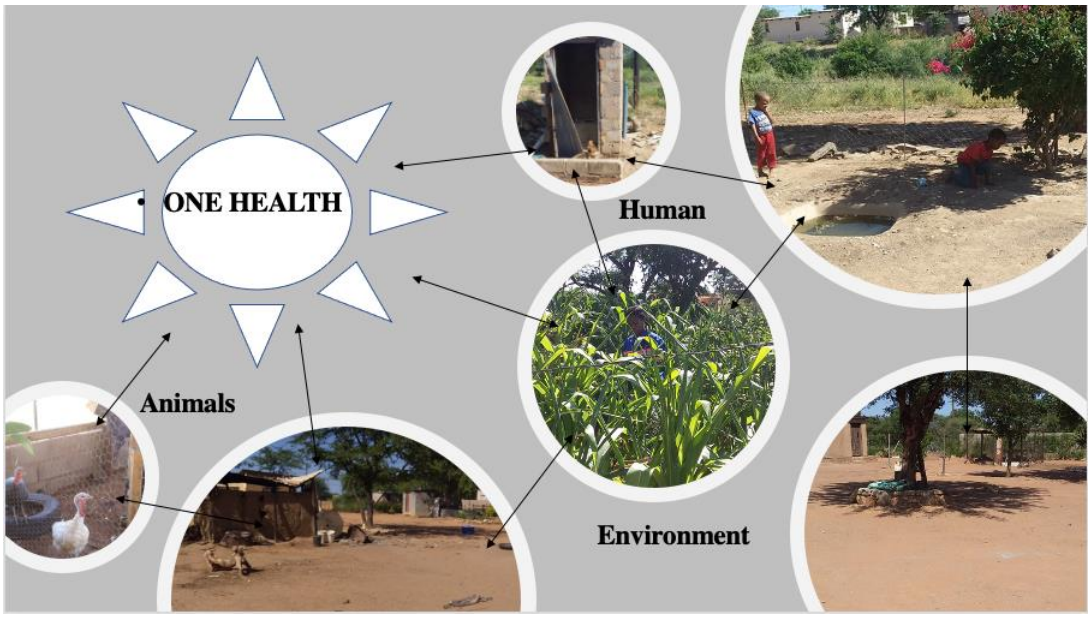


Figure 3.1.B: One health setup showing the possible interactions of human, animal, and environment in the peri urban Palapye settlement (©KBrooks).

3.2 Sample Collection

The various samples collected from the site include feces samples from three different domesticated animals (such as chicken, dogs, ducks), soil samples from three different spots (compound, outside and garden soil) and pond water where all these animals drank, and children played (Figure 3.2.A).

All samples were collected in triplicates in the morning between 8 am and 10 am in March 2020. Soil samples were collected using a spatula sterilized with 70 % ethanol between samplings, 2-5 cm beneath the surface into a sterile Ziploc bag. Animal feces samples were collected from the ground using a sterile forceps into sterile 15 mL falcon tubes, and pond water was collected in sterile plastic screw-capped bottles by dipping them in the pond. Approximately 50 g of soil and feces samples and 250 mL of pond water were collected, kept in a cooler with ice packs, and transported to the laboratory within 12 h and instantly subjected to microbiological analysis. Each sample was collected in triplicate.

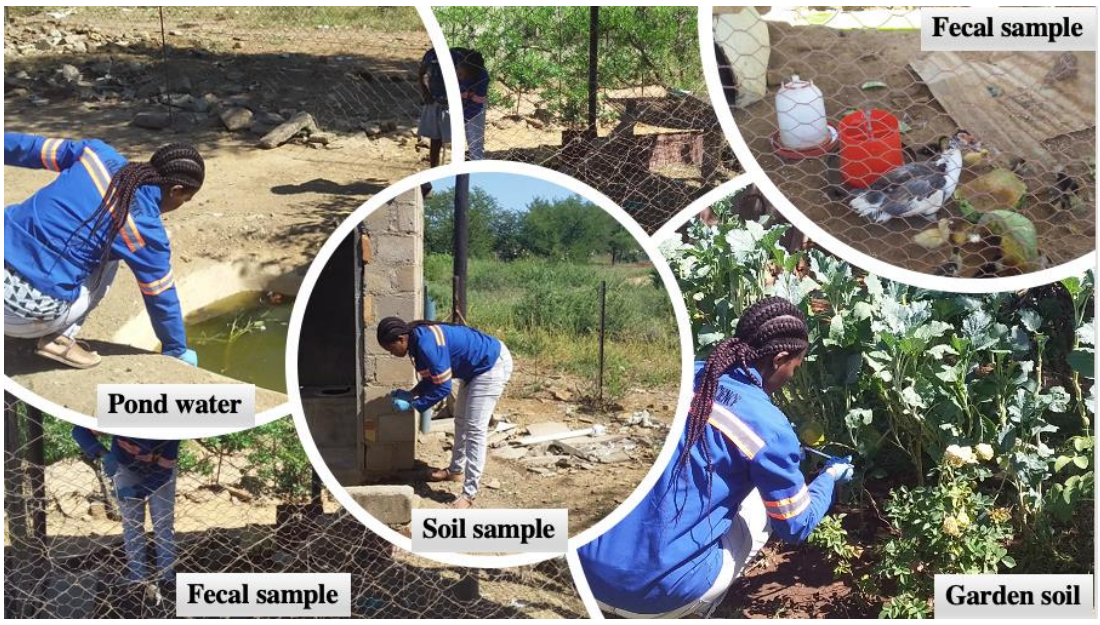


Figure 3.2.A: Types of samples collected from the sampling sites (©KBrooks)

3.3 Experimental design

The experimental workflow shown below (Figure 3.3.A) explains how to isolate, identify bacteria, and classify antibiotic resistance determinants using "wet lab" (*in vitro* analysis) and "*in silico* analysis" in this study. The wet lab step is shown as the isolation of antibiotic-resistant bacteria in accordance with the study's expected objective, followed by extraction of genomic DNA from culture and extraction of community DNA from uncultured samples. The last step in wet lab analysis is next-generation sequencing, which generates raw reads for the next important phase, which is bioinformatics analysis (*in silico* analysis). In the bioinformatics step, the raw reads were assembled in order to obtain genomes and thereafter characterized to predict taxonomy of the microbial community in uncultured samples and identify bacterial genomes in cultured samples using several accessible bioinformatics tools and programs. Furthermore, the assembled reads were also examined for antibiotic resistance, plasmid, and virulence genes.

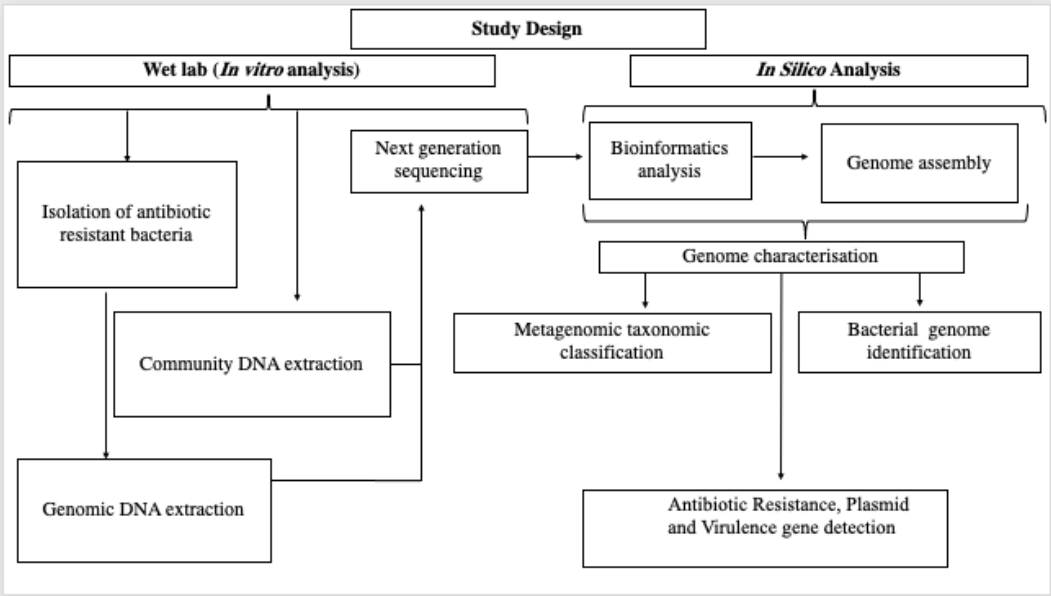


Figure 3.3.A: Experimental workflow of the study. The study's workflow illustrates the major two methods: wet lab (*in vitro* analysis) and *in silico* analysis, with steps at each stage.

3.4 Isolation of antibiotic resistant bacteria

Samples from soil, animal feces and pond water were analyzed for the isolation of bacteria on different selective and differential agar media which targeted different bacterial species. Approximately 1 g of soil, feces, and 1 mL of pond water were pre-enriched in a non-selective 9 mL of tryptic soy broth (TSB) to allow for a wide variety of microorganisms to grow for 24 h at incubation of 37 °C with agitation. Approximately 0.1 mL of the turbid broth was serially diluted (dilutions 10^{-1} to 10^{-5}) and spread plated in triplicates using a sterile glass spreader onto MacConkey agar [BioLab, Merck, (South Africa)] and Mannitol Salt Agar [Lab M, Neogen, (United Kingdom)] at 37 °C for 18 to 24 h. Colonies that grew on MacConkey agar plate and Mannitol Salt agar were examined and randomly selected. Of all the bacterial isolates counted, 336 isolates were selected as a result of picking an average of 8 morphologically different bacterial isolates from triplicate plates of each different sample source from MacConkey and Mannitol salt agar media. The isolates were then further stored into duplicate 96 well plate containing nutrient broth with 50 % glycerol (ratio 1:1) and stored at – 80 °C for further antibiotic resistance susceptibility test and DNA extraction.

3.5 Antibiotic susceptibility test

To determine antibiotic resistance profiles, bacterial isolates stored in glycerol were analyzed using the agar dilution method, which involves prepared media supplemented with various antibiotics at different concentrations adopted from clinical breakpoint concentrations as previously described by Tapela and Rahube (Tapela & Rahube, 2019). This method enables screening of substantial number of isolates, and it is most suitable for surveillance testing and research. Briefly, the frozen bacterial isolates were thawed at room temperature, picked using sterile toothpicks from the storage tube containing the bacteria and glycerol, and aseptically streaked using the sterile toothpicks into 50 grid squared nutrient agar containing the different antibiotics and each bacterial isolate was aseptically sub-cultured into several 50-grid squared nutrient agar plate each containing different antibiotic concentrations;

penicillin (16 µg/mL), ampicillin (32 µg/mL), cephalosporin (32 µg/mL), meropenem (4 µg/mL), cefotaxime (64 µg/mL), cefoxitin (32 µg/mL) . cycloheximide (75 µg/mL) [Sigma-Aldrich Co., St. Louis, MO, USA] was added in the preparations of the media to control fungal growth on the media plates. Clinical breakpoint concentrations used in hospitals for the treatment of bacterial infections were utilized to determine the antibiotic concentrations. The controls included nutrient agar media without antibiotics and a sensitive strain of *E. coli* DH5α. The susceptibility of the isolate to the antibiotic was determined by the observation of visible growth as the presence (Resistant) and absence (Sensitive) to the antibiotic following incubation of plates at 37 °C for 48 h.

3.6 DNA Extraction

3.6.1 Genomic DNA extraction

DNA from cultured bacteria (volume of 1.5 mL) was extracted using the method previously described by Tapela and Rahube (2019), with few modifications. A thawed bacteria from dog feces isolate which was stored in – 80 °C freezer was enriched in nutrient broth by incubation at 37 °C for 24 h with agitation. The overnight culture cells were harvested by centrifugation at 13000 x g for 1 min. Then the pellet cells were briefly suspended in 600 µL of cell lysate solution (500 mM Tris-HCl, 200 mM EDTA, 460nM NaOH; pH8.0 and 15% SDS pH 6.6), the mixture was gently mixed by inversion and incubated at 80 °C in a water bath for 20 min. Next 20 µL of Proteinase K was added and then incubated at 56 °C for 20 min. DNA was purified by adding 200 µL of phenol and chloroform/isoamyl alcohol, then followed by 200 µl of chloroform and the mixture was vortexed for 20 s. The mixture was then centrifuged at 13, 000 rpm for 3 min to pellet the protein. The upper aqueous layer containing the DNA was transferred into a new tube then 600 µL of Isopropanol was added. The mixture was centrifuged at 13,000 rpm for 30 min to obtain the DNA pellet. The obtained DNA pellet was then washed with 500 µL cold 70 % ethanol and air dried.

Finally, 100 µL TE buffer (10 mM Tris-HCl, pH8.0, 1 mM EDTA) was added to the DNA pellet and stored for subsequent analysis.

3.6.2 Community DNA extraction

For bacterial community analysis, total community DNA was extracted using Quick-DNA Feces/Soil Microbe kit (Zymo Research, United States of America) according to the manufacturer's instructions directly from the uncultured samples (directly from the uncultured samples (animal feces, soil and pond water samples. 0.25 g of soil/animal feces was weighed and added into a ZR BashingBead^{TM} Lysis Tube (0.1 mm) whilst for water sample, 250 mL of water was filtered through a 0.45 µm pore size membrane filter paper which was cut into small pieces by sterilized scissors and placed into ZR BashingBead Lysis tubes (0.1 mm). For both soil, animal feces, and water, the DNA was extracted following the manufacturer's instructions. The extracted DNA was evaluated for DNA yield and quality using a Nanodrop one spectrophotometer (ThermoFisher scientific, United States of America) at an absorbance of 260 nm and the DNA was stored at -20 °C prior to DNA sequencing.

3.7 Next Generation sequencing

The genomic DNA sample extracted from cultured bacteria from dog feces sample (WGS004) and community DNA samples animal feces (F001), pond water (P002) and environmental soil (S003) were subjected to shotgun sequencing on the Illumina MiSeq platform at Inqaba Biotechnical Pty Ltd, South Africa. Briefly, DNA samples were fragmented using an enzyme-based approach following the protocol from New England BioLab's Next Ultra II kit^{TM}. Resulting fragments were purified (size selected), end-repaired and an Illumina specific adapter sequence was ligated to all fragments. The samples were quantified, individually indexed followed by a second size selection step using AMPure XP Beads. The libraries were quality controlled on a DNA chip (Agilent 2100 Bioanalyzer) and then sequenced using a MiSeq v3 (600

cycle) kit according to the manufacturer's protocol. The sequenced libraries generated the raw reads in fastq format which were further analyzed using bioinformatics tools.

3.8 Bioinformatics Analysis

3.8.1 Genome assembly

The raw paired ends reads from dog feces cultured bacteria genome sequencing (WGS004), and metagenomic sequencing (animal feces, pond water, and environmental soil) were uploaded into Pathosystems Resource Integration Center (PATRIC) assembly tool ((https://patricbrc.org/app/Assembly2)) for *de novo* assembly performed using Unicycler (v0.4.8) program (Wick et al., 2017), which incorporates the trimming process of the reads using TrimGalore, polishing tool (Pilon v1.23) and quality check tool (QUAST (v4.6.0)) to correct assembly errors and quality.

3.8.2 Bacterial genome identification

After assembly of the reads from cultured bacteria genome sequencing, the contigs were uploaded on the web-based server of Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/services/KmerFinder/) under the KmerFinder tool which identifies the closely related bacterial strains found in the bacterial genome sequences. The scoring system for KmerFinder (version 3.2) (Larsen et al., 2014) provides results as a summary of the number of *K*-mers that matched each template with the highest score and the default settings were based on "standard scoring method" settings (https://cge.cbs.dtu.dk/services/KmerFinder/output.php) (Hasman et al., 2014). Furthermore, The KmerFinder output generated the predicted closely related reference genomes to the contigs of the dog bacterial genome which served as a guide for contig alignment and visualization against references using the CGviewer (Circular Genome Viewer) server (http://stothard.afns.ualberta.ca/cgview_server/) (Grant & Stothard,

2008) through NCBI BLAST comparison. Lastly, PATRIC Comprehensive Genome Analysis Service tool was used to determine the phylogenetic relatedness of the predicted species. PATRIC program employed Mash/MinHash (Ondov et al., 2016) to offer NCBI references and representative genomes closely linked to the sequenced dog feces sample (WGS004). The PATRIC global protein families (Davis et al., 2016) were then chosen from these genomes to assess the genome's phylogenetic position. MUSCLE (Edgar, 2004) was used to align the protein sequences from these families, and the nucleotide alignments were concatenated into a data matrix, which RaxML (Stamatakis, 2014) was used to analyze, with fast bootstrapping (Stamatakis et al., 2008) employed to construct the support values in the tree. All the tools used to generate the phylogenetic tree were embedded in the comprehensive genome analysis tool.

3.8.3 Metagenomic taxonomic classification

Shotgun metagenomics reads were uploaded to the PATRIC taxonomic classification tool (https://patricbrc.org/app/TaxonomicClassification) for taxonomic classification (species composition). This tool utilizes the Kraken 2 program to assign reads to taxonomic labels through precise *K*-mer alignment, providing an initial microbial composition present in a sample (Wood & Salzberg, 2014). The Kraken 2 results from PATRIC were presented in pie charts using Microsoft excel to show the diversity and abundance of the microbial community within the metagenomics reads of animal feces, pond water and soil. The pie chart information was retrieved from the Kraken based species outputs and krona charts with their ancestries line obtained from the NCBI taxonomy database.

3.8.4 Antibiotic Resistance, Plasmid and Virulence gene detection

The identification of acquired and chromosomal ARGs genes were detected using the ResFinder tool (version 4.1) (https://cge.cbs.dtu.dk/services/ResFinder/) in Center for

Genomic Epidemiology website (Zankari et al., 2012; Bortolaia et al., 2020). The default parameters were used with threshold of minimum length coverage of 60 % and minimum identity of 90 % (Bortolaia et al., 2020). In addition, Comprehensive Antibiotic Resistance Database – CARD (Alcock et al., 2019) was also employed to search for AMR genes. Two other resistance gene databases; PATRIC metagenomics read mapping service (https://patricbrc.org/app/MetagenomicReadMapping) and PATRIC Comprehensive Genome Analysis Service along with annotation with RAST tool kit (RASTtk) algorithm (Brettin et al., 2015) embedded in PATRIC tool, utilized homology to known sequences to identify genes associated to antibiotic resistance genes. This tool uses the k-mer-based AMR gene detection method, which uses PATRIC's curated library of typical AMR gene sequence variants (Wattam et al., 2016).

The presence of plasmids were predicted using PlasmidFinder tool (version 2.1) (https://cge.cbs.dtu.dk/services/PlasmidFinder/) with default parameters of identity of 95% threshold and 60 % minimum length coverage (Carattoli, et al., 2014). VirulenceFinder (version 2.0) (https://cge.cbs.dtu.dk/services/VirulenceFinder/) was used for the identification of known virulence genes (Joensen et al., 2014) with default parameters of minimum length coverage of 60 % and minimum identity of 90 % (Bortolaia et al., 2020) .

3.8.5 Nucleotide Sequence Accession Numbers

The bacterial shotgun genome sequencing and metagenomics shotgun sequencing data in this study have been deposited at NCBI database under BioProject accession number PRJNA772288. The following accession numbers can be found under this BioProject link (https://www.ncbi.nlm.nih.gov/sra/PRJNA772288): SRX13081222, SRX13081223, SRX13081224, and SRX13081225.

CHAPTER 4:RESULTS

4.1 Phenotypic β-lactam resistance

There was a total of 21 samples represented by pond water (n=3), animal feces (n=9), and environmental soil samples from different surroundings of the household (n=9). A total of 336 isolates, where (42.9 %) were from different animal feces, (42.9 %) were from surrounding soil samples, and (14.2 %) were from pond water.

Based on the ß-lactam resistance phenotypes of the isolates from animal feces, pond water and soil sources, there was resistance to all the ß- lactam antibiotics across all 336 isolates where animal feces, surrounding soil samples and pond had 100 % resistance to penicillins (penicillin and ampicillin), 100 % resistance to cephalosporins (Cephalosporin, Cefoxitin, 3rd generation cephalosporin: cefotaxime) and 100 % resistance to carbapenem (meropenem) respectively (Figure 4.1.A).

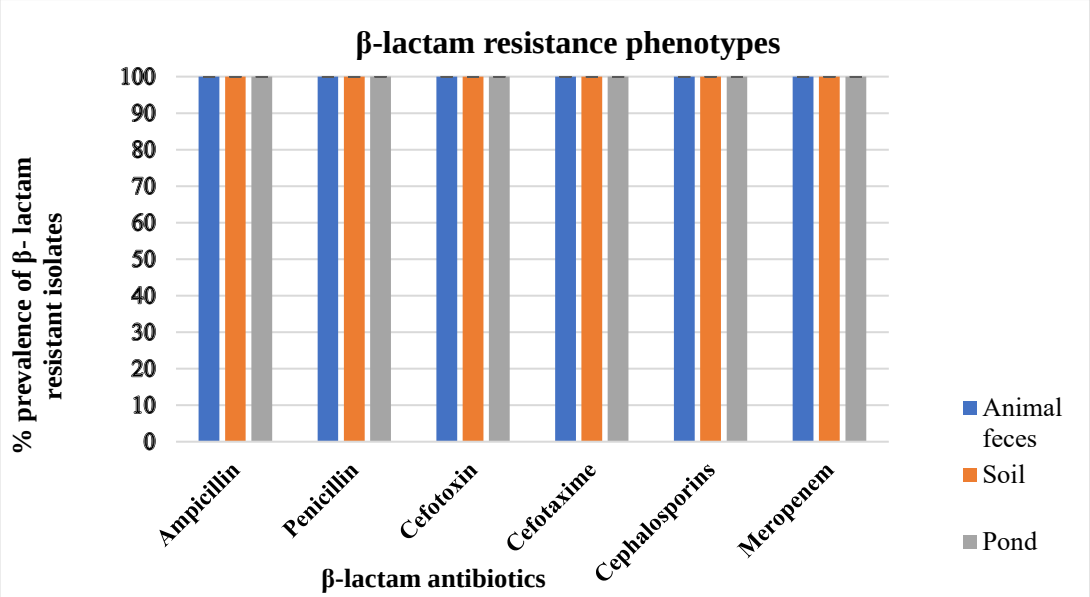


Figure 4.1.A: Phenotypic ß-lactam resistance prevalence of isolates from different sources.

4.2 Bacterial genome shotgun sequence

The sequenced dog feces cultured bacteria sample (WGS004) assembly revealed a total size of 7, 745,935 bp with a G + C content (36.92 %), total number of contigs (2017) the largest contig was 162722 bp (Table 4.2-1).

Table 4.2.1: Summary of statistics for the sequence data from dog feces sample

Sample Name	**No. of contigs**	**Largest contig**	**Total assembly size**	**GC %**	**N50***
Dog feces (WGS004)	2017	162,722	7, 745,935	36.92	6,984
*N50: this value denotes the length of the shortest sequence in the assembled genome at 50% (halfway of the genome).					

4.2.1 Bacterial identification and phylogenetic analysis

Bacterial species and strains were identified in the dog feces sample, indicating the sample to be a mixed culture. The closely related identified strains included *Proteus mirabilis* strain (CRPM10), *Citrobacter* sp. (RHB21-C05), *Paeniclostridium sordellii* strain (AM370), *Proteus mirabilis* strain (AR_0059) and *Proteus mirabilis* strain (PmSC1111), respectively. According to the predicted species, the most dominant species is shown to be *Proteus mirabilis*, followed by *Citrobacte*r species and *Paeniclostridium sordellii* species. This KmerFinder output **(**see **Appendix)** is supported by NCBI BLAST sequence similarities observed in the CG viewer below (Figure 4.3.A).

In comparison to the predicted reference species, the alignments revealed areas of similarity and distinct regions of dog feces contigs. The greatest homology BLAST hits linked to the dog feces contigs were found in the *Proteus mirabillis* species. As seen in Figure 4.3.A, each genome is represented by a specific color, with darker sections in the circular genome indicating the strongest BLAST sequence similarity to the predicted reference genome and lighter portions indicating lower BLAST sequence similarity matches. The map also showed several white gaps between the

predicted reference genomes and the dog feces contigs that had no BLAST hits, indicating no homology to the reference genomes and dog feces contigs.

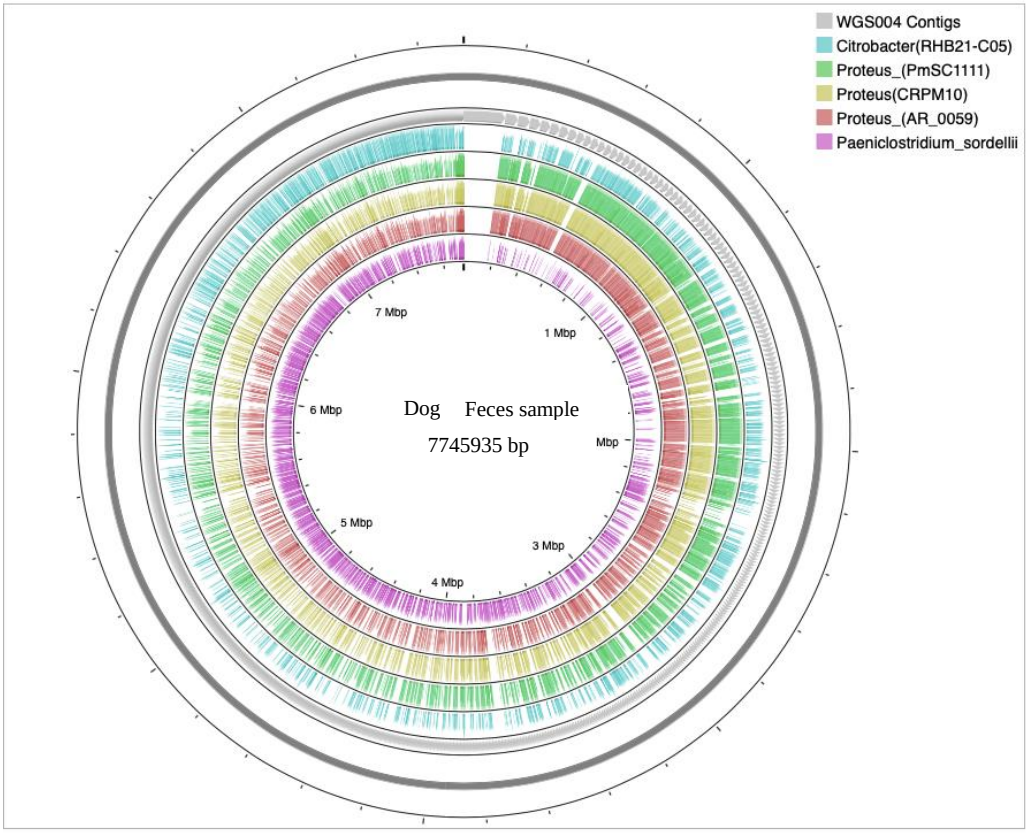


Figure 4.2.A: A generated map using the CGviewer showing a full view of dog feces contigs with BLASTN reference genome comparisons. The contents of the feature rings (starting with the outermost ring) are as follows. Ring 1: indicates the primary sequence reads from dog feces contigs (WGS004); Ring 2: *Citrobacter sp* (RHB21-C05); accession: NZ_CP057611.1; Ring 3: (*Proteus mirabilis* strain (PmSC1111) accession: NZ_CP034090.1; Ring 4: (*Proteus mirabilis* strain (CRPM10) accession: NZ_CP043332.1; Ring 5: (*Proteus mirabilis* strain (AR_0059) accession: NZ_CP020052.1 and Ring 6: (*Paeniclostridium sordellii* strain) accession: NZ_CP014150.1 showing the BLAST coverage and comparison results (BLASTN) with dog feces contigs (WGS004); The BLAST coverage and comparison results are shown with shading, with darker portions indicating multiple and highest hits to the

relevant portion of the reference sequences whereas white gaps reveal areas with no similarity to the predicted reference genomes.

The maximum likelihood phylogenetic tree of the dog feces sample revealed that the dog sample contains various bacterial species closely related to *Proteus mirabillis* genus, the *Citrobacter* genus, and *Clostridium sordelli* also known as *Paeniclostridium sordelli*, confirming the relationship and similarity of the dog feces sample with the predicted reference genomes. According to phylogenetic analysis of the dog feces contigs (Figure 4.2.B), there are two major clades. Clade A is made up of several *Clostridium* species, while clade B is made up of *Shigella, Citrobacter*, and *Proteus* species. In clade B, the closest genomic sequence from NCBI belonged to the *Proteus mirabilis* strain (HI4320 529507.6), which had the highest proximity to dog feces sample. This suggests that the genomic sequence was more closely related to the *Proteus* genus. Other strains in the closest taxonomic group include *Proteus penneri* (ATCC 35198 471881.3), *Citrobacter freundii* (CFNIH1 1333848.3), and the furthest is represented by *Clostridium sordellii* (VPI 9048 1292035.3).

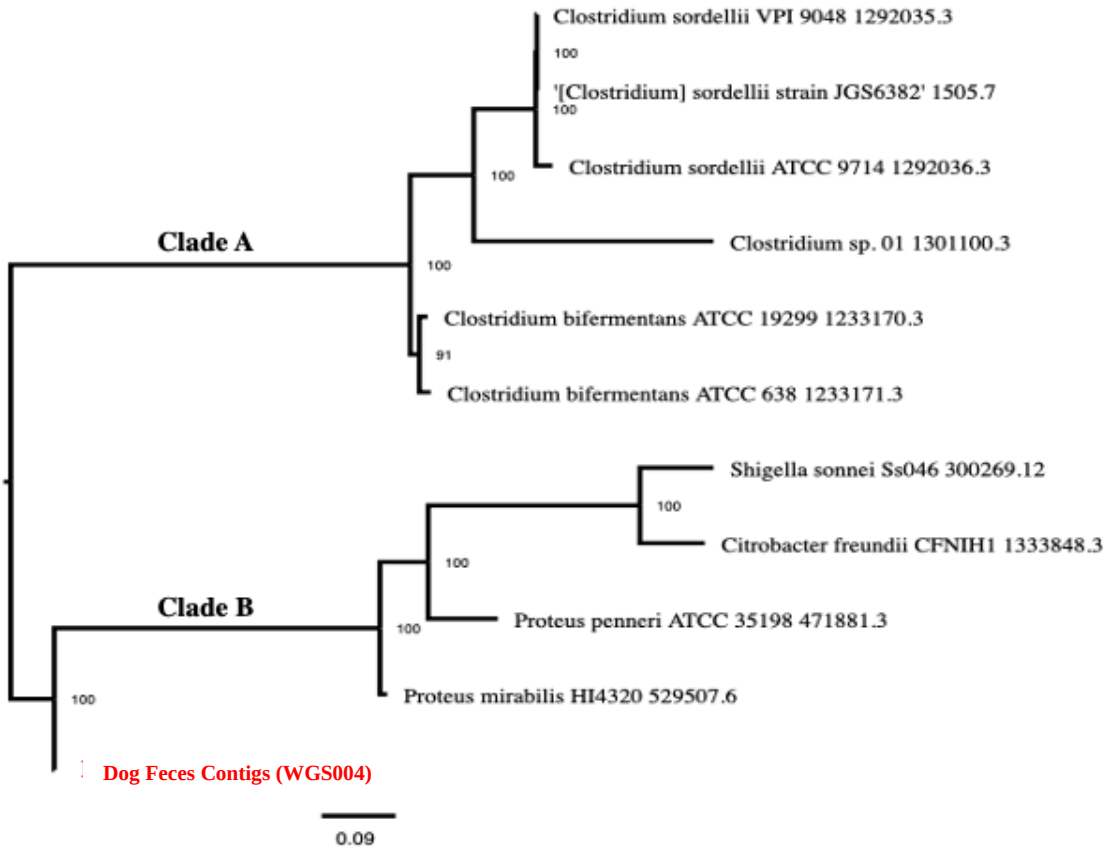


Figure 4.2.A: The phylogenetic tree depicts the closest taxonomic relative of dog feces contigs (WGS004). The maximum Likelihood phylogenetic tree is based on the PATRIC global protein families within dog feces sample (in red) along with other well studied counterparts from NCBI (in black).

4.2.2 AMR gene identification from dog feces sample

Various gene determinants of antibiotic resistance and AMR pathways were discovered through a thorough genomic investigation of a dog feces sample. Table 4.2.2 summarizes the different acquired and chromosomal mutational ARGs found in dog feces, as well as the various mechanisms associated with them. The majority of the ARGs found in the dog feces were linked to chromosomal mutations and conferred resistance to a wide range of antibiotics classes. The least discovered ARGs were associated with ß- lactam antibiotic class.

Table 4.2.2: AMR gene identification from dog feces sample

Antibiotic class	**AMR Mechanism**	**Genes**
Multidrug	Antibiotic Efflux	*Acr*AB-TolC, *Acr*AD-TolC, *Acr*Z, *Emr*AB-TolC, *Emr*D, *Mac*A, *Mac*B, *Mdt*ABC-TolC, *Mdt*L, *Mex*HI-OpmD, *Sug*E, *Tol*C/*Opm*H
Tetracycline		*Tet*(J), *Tet*A(P)
Phenicol	Antibiotic inactivation	*cat*A1/*cat*A4
	Antibiotic activation	*Kat*G
Multidrug	Antibiotic target alteration	*Alr, Ddl, dxr, EF-G, EF-Tu, fo*lA, *Dfr, fo*lP, *gyr*A, *gyr*B, *inh*A, *fab*I, *Iso*-tRNA, *kas*A, *Mur*A, *rho*, *rpo*B, *rpo*C, *S10*p, *S12*p, *Gdp*D, *Mpr*F, *Pgs*A,
ß- lactam		*fts*I
Tetracycline	Antibiotic target protection	*tet*B(P)
Multidrug	Antibiotic target replacement	*Fab*
Multidrug	Antibiotic target deletion	*gid*B

Multidrug class includes ß-lactams, fluroquinolone, phenicol, macrolide, aminoglycosides, folate pathway antagonist (trimethoprim) and Fosfomycin followed by tetracycline and phenicol drug classes

4.3 Metagenomics shotgun sequence

The assembly summary of the three metagenomic sequences are shown in table 4.3.1. Pond water sample had the highest total read size (25.8 Mb) and the largest number of contigs (11,437 bp), with 48.83 % GC content, animal feces followed by total read size of 8,465,934 bp with 3893 contigs and GC content of 47.22 %. Finally, when compared to other assembled samples, soil sample had the smallest number of contigs (34), the smallest assembly size (68.4 kb), and the highest G + C content of 51.44 %.

Table 4.3.1: Summary of statistics for the sequence data from different sources

Sample ID	**Number of contigs**	**Largest contig**	**Total assembly size**	**GC %**	**N50**
Pond water	11,437	93,027	25,777,472	48.53	3811
Animal feces	3,893	53,991	8,465,934	47.22	2549
soil	34	6,925	68,409	51.44	2083

*N50: this value denotes the length of the shortest sequence in the assembled genome at 50% (halfway of the genome).

4.3.1 Taxonomic identification

The taxonomic identification from the metagenomic sequences revealed diverse microbial communities from the different samples. The results of the metagenomic taxonomic profiles were compared at the domain level including viruses. Domain bacteria was further presented and compared at the classification levels of phylum, class, order, family, and genus profiles.

4.3.1.1 Domain profile

All three samples had =/> 96 % sequences identified as bacteria, the pond sample has 98 % of bacteria as compared to feces sample and soil with 96 %. The other noticeable domain is in soil sample which is the Eukaryota (3 %) while in other samples only 1 % of Eukaryota is observed in feces and pond samples. Viruses were detected mostly in the feces (3 %) and pond (1 %), while soil sample revealed only 0 %. Apart from bacteria and Eukaryota, the archaea domain was found in a small percentage of soil samples (1%) but not in feces or pond water sample.

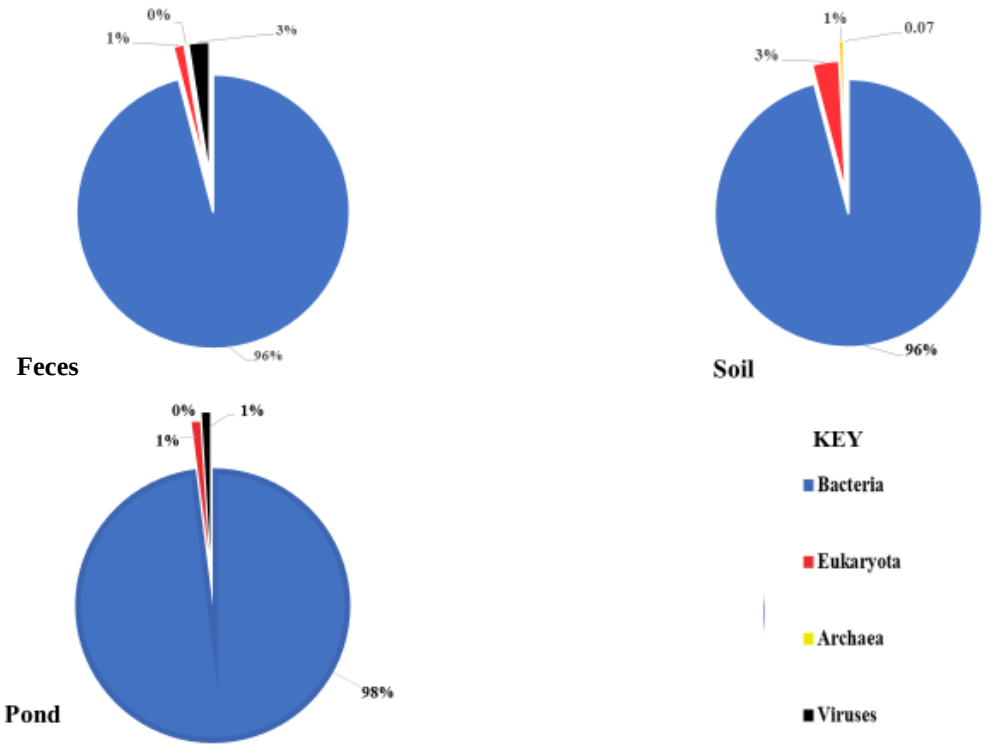


Figure 4.3.A: Domain profiles for feces, pond and soil sample

4.3.1.2 Bacterial Phylum profile

As shown in Figure 4.3.A, bacterial DNA was the most prevalent in all the samples, so it was further investigated at the phylum level. The bacterial phylum profile revealed that the Proteobacteria phylum is the most abundant bacteria phylum across all samples, with the pond sample having the highest relative abundance of 78 %, followed by feces with 64 %, and soil with 50 %. Actinobacteria is found in greater abundance in soil samples (43 %), compared to feces (23 %), and pond water (17 %), respectively. The Firmicutes phylum was represented with 5 % in feces, 4 % in pond, and 3 % in soil samples. The Chordata and Bacteroidetes phyla have the lowest abundance, with only <=7 % across all samples (Figure 4.4.B).

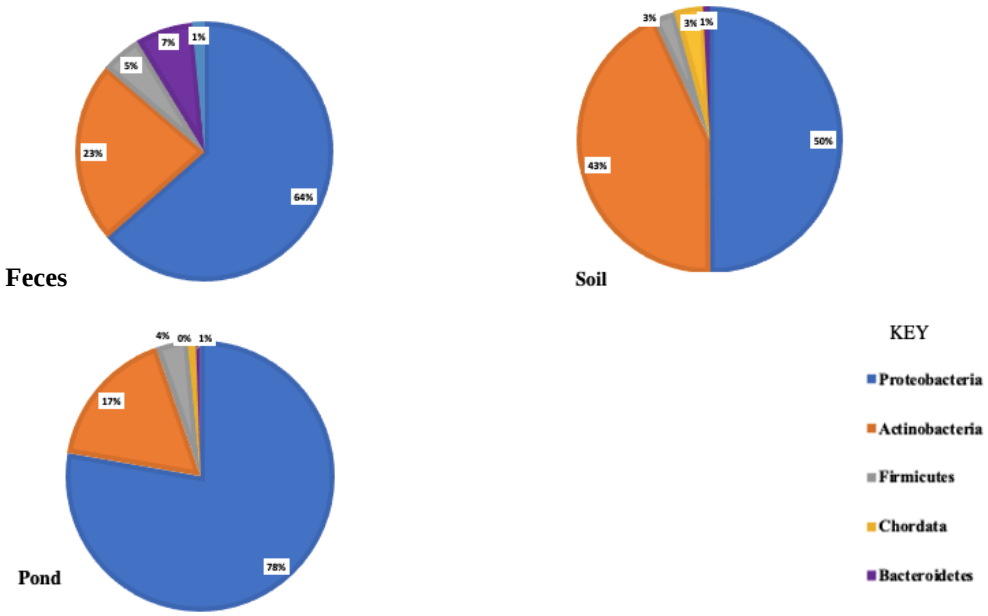


Figure 4.3.B: Phylum profiles for feces, pond and soil samples

4.3.1.3 Class profile

Proteobacteria was found to be the most common in all the samples, as shown previously (Figure 4.3.B), thus it was explored further at the class level. Figure 4.4.C shows that the most abundant class profile across all samples is Gamma proteobacteria, with the feces sample leading with 71 %, followed by the pond sample with 68 %, and the soil with 42 %. Alpha proteobacteria are most abundant in soil samples, accounting for 36 %, while other samples had less than 6 % relative abundance. Delta proteobacteria was also found to be abundant in pond and feces samples, with 13 % and 12 % respectively, while the soil sample had the least, with just 7 %. The abundance of betaproteobacteria in the soil and feces was 15 % and 13 %, respectively, while the pond only had a 5% abundance.

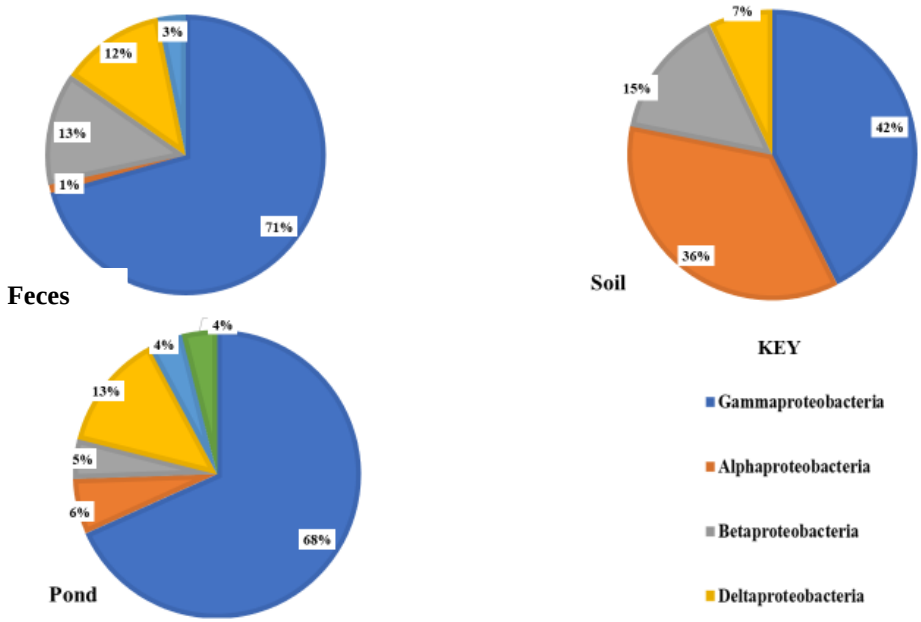


Figure 4.3.C: Class profiles for feces, pond and soil samples

4.3.1.4 Order profile

As shown in Figure 4.3.D, the Enterobacterales were the most abundant order profile, which is from the Gamma proteobacteria class, accounting for 69 % and 62 % of the pond and feces samples, respectively, with soil accounting for just 22 %. The succeeding order was represented by Steptomycetales, which had the highest percentage in soil (20 %), followed by pond sample (11 %), and feces sample (7 %), respectively. Rhodobacterales was the least abundant order profile in soil and pond, with 9 % and 3 %, respectively, and other order profiles such as Corynebacterlales and Micrococcales were found to be less abundant across all different samples.

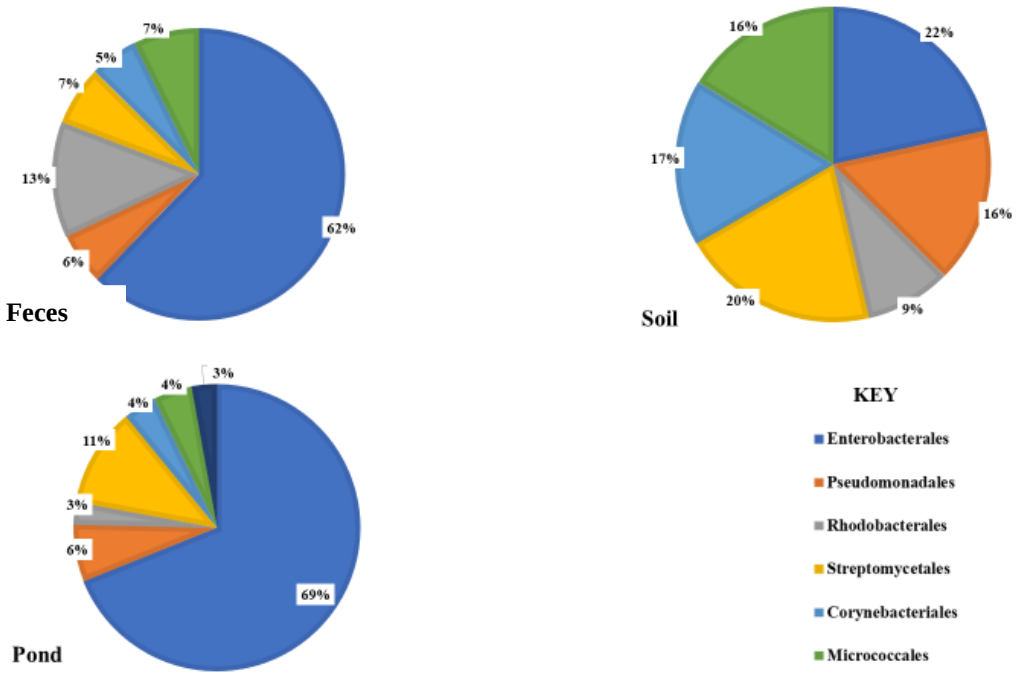


Figure 4.3.D: Order profiles for feces, pond and soil samples

4.3.1.5 Family profile

At family level, the most abundant family was *Enterobacterales*, which was found in 58 % of pond and 56 % of feces samples, respectively, with soil being the least abundant at 24 %. The Streptomycetacea family showed the largest abundance in the soil sample, accounting for 32 %, followed by 4 % in both the pond and feces samples. The Morganellaceae family had a 24 % relative abundance in both pond and feces, whereas soil had relative abundance of 9 %. Other less abundant family profiles (<=6 %) such as Pseudomonadaceae, Streptomycetaceae, and Mycobacteriaceae were represented in feces and pond, respectively, with 22 % and 13 % for Pseudomonadaceae and Mycobacteriaceae in soil (Figure 4.3.E).

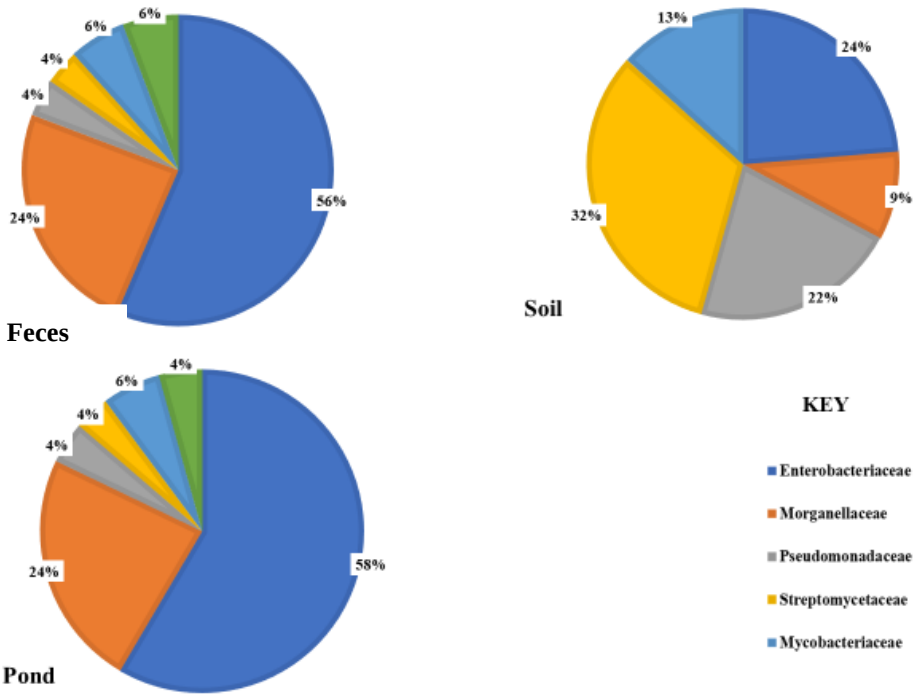


Figure 4.3.E: Family profiles for feces, pond and soil samples

4.3.1.6 Genus profile

At genus level, *Klebsiella* was the most abundant genus across all profiles, accounting for 44 % in both pond and soil, with feces accounting for 49 %. The *Proteus* genus was found in feces (42 %), soil (40 %), and pond (39 %) respectively. As indicated in Figure 4.3.F, *Escherichia* and *Enterobacter* were the least abundant genus in all samples, with feces (4 %, 10%), pond (4 %, 8%), and soil (8 %) accordingly.

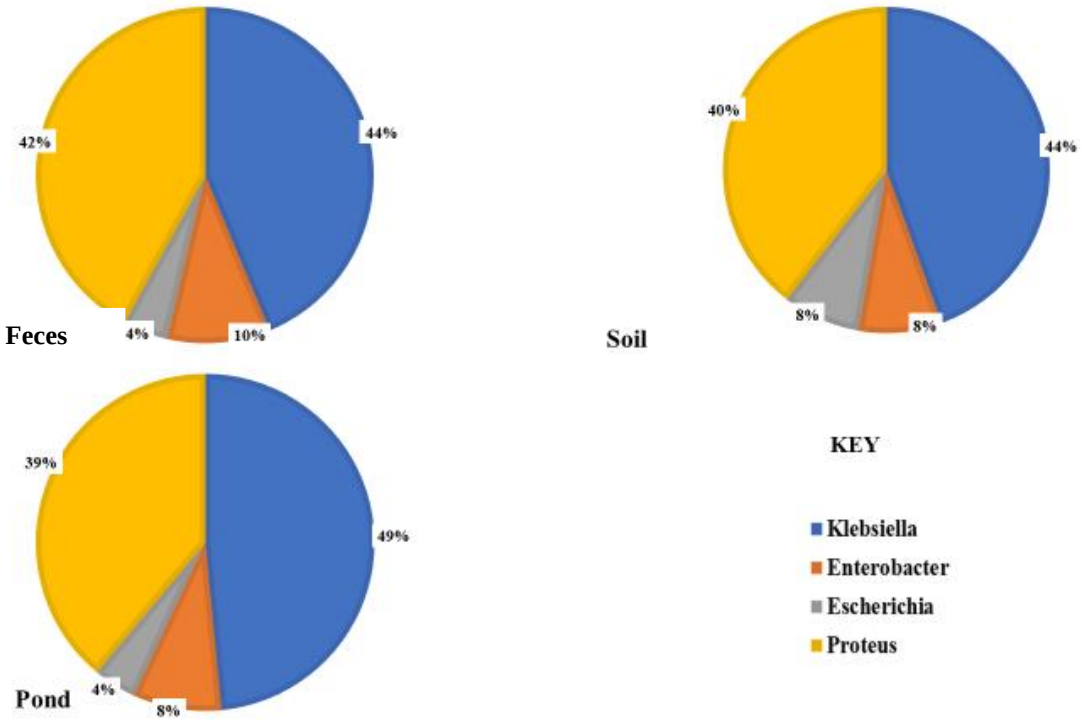


Figure 4.3.F: Genus profiles for feces, pond and soil samples

4.3.2 AMR gene identification from different sources

The acquired antibiotic resistance genes profiles of animal feces, pond water and environmental soil are compared in Table 4.3.2. Several types of ESBL genes encountered in animal feces included different variety of SHV (*bla*$_{\text{SHV-82}}$, *bla*$_{\text{SHV-81}}$)

followed by *cfx*A (*cfx*A2, *cfx*A6), TEM ($bla_{\text{TEM-208}}$) and SED ($bla_{\text{SED1)}}$. In comparison to pond water sample, there was diverse occurrence of ESBLs genes which included four SHV variants ($bla_{\text{SHV-11,}}$ $bla_{\text{SHV-81}}$, $bla_{\text{SHV-180}}$, $bla_{\text{SHV-172}}$), two OXA variants ($bla_{\text{OXA-217,}}$ $bla_{\text{OXA-50}}$), TEM ($bla_{\text{TEM-206}}$), and other ß-lactamase resistance genes which were LEN ($bla_{\text{LEN-24}}$), CMY ($bla_{\text{CMY-98)}}$, SED (bla_{SED1}) and OKP-B ($bla_{\text{OKP-B-17}}$). These genes are responsible for the production of β-lactamase enzymes and resistance to β-lactam antibiotics. In soil samples, there was no acquired ESBL gene discovered.

Table 4.3.3 also shows chromosomal mutation resistance genes encoded for the outer membrane porins (*Omp*K35, *Omp*K36, *Omp*K37) associated with β-lactam antibiotics like cephalosporins and carbapenems across all the different samples. Other point mutation genes discovered in animal feces and pond water included *amp*C, *Omp*C and *Omp*F. In addition, pond water had *opr*D and *omp*36 resistance genes that also reduce permeability to antibiotics (β-lactams) or cause resistance by absence (deletion).

The most abundant non ß-lactamase genes found in animal feces isolates were represented by diverse variants of *tet* genes conferring resistance to tetracycline (*tet*(W), *tet*(40), *tet*(A), *tet*(J), *tet*Q, *tet*A(P), *tet*R, *tet*(W/N/W), *tet*X, *tet*(L), *tet*O, *tet*B(P), *tet*(D)), aminoglycosides genes (*aph*(6)-Id, *aac*(6')-Iid, *aac*(2')-Ia, *aph*(3")-Ib) and phenicol (*cat*, *cat*III) while for pond water, the diverse *tet* genes revealed included (*tet*(A), *tet*(J), *tet*A(P), *tet*R, *tet*(L), *tet*(D), *tet*B(P)) and different variants of aminoglycosides genes (*aph*(6)-Id, *aac*(6')-Iid, *aac*(2')-Ia, *aph*(3")-Ib, *aph*(3)-IIb, aac(6')-Ii, *aac*(6')-Iz) and phenicol genes (*cat*, *cat*II, *cat*III, *cat*B7). In comparison to soil, the AMR genes detected were also *tet* gene variants (*tet*(J), *tet*(L)) conferring resistance to tetracycline class, aminoglycosides (*aph* (6)-Id, *aph*(3")-Ib) and phenicol (*cat*) conferring resistance to chloramphenicol. Other antibiotic resistance genes providing resistance to phenicol (*cat*II, *cat*III, *cat*B7), fluoroquinolone (*oqx*A, *oqx*B), macrolide (*mef*A) fosfomycin (*fos*A, *fos*A2, *fos*A3, *fos*A5, *fos*A6), trimethoprim-sulfamethoxazole (*dfr*A14, *dfr*A15) and sulfonamide genes (*sul*1, *sul*2) were detected across all the different samples in addition to acquired resistance.

Multiple drug class genes conferring resistance to a wide variety of antibiotics (ß-lactams, fluroquinolone, phenicol, macrolide, aminoglycosides, trimethoprim, fosfomycin and sulfonamide) were found in abundance across all samples as shown in Table 4.3.3. These resistance genes were mostly chromosomal point mutation genes that represented various AMR mechanisms such as antibiotic efflux (*emr*R, *emr*A, *emr*K, *emr*E , *emr*Y, *emr*B, *acr*A, *acr*B, *acr*E, *acr*F,*acr*R, *acr*S, *ade*K, *ade*I, *ade*B, *ade*J, *ade*F, *ade*H, *ade*G, a*de*N, *ade*L, a*de*A, *ade*I, *ade*S, *ade*R, *efr*A, *efr*B, CRP, *mdf*A, *nal*C, *nal*D, *ram*A and *mex*P) conferring resistance to fluoroquinolone and multiple drug classes, antibiotic target alteration (*par*C, *parE*, *gyr*A, *gyr*B, *Uhp*T, *Cya*A, *Uhp*A, *Glp*T, *mur*A, *Pts*I and *fol*P) conferring resistance to fluoroquinolone, fosfomycin, sulfonamide and antibiotic target replacement (*dfr*E, *dfr*F and *dfr*G) conferring resistance to trimethoprim-sulfamethoxazole.

Table 4.3.2: Acquired AMR genes from different sources

Class	**Genes**	**AMR Mechanism**	**Animal feces**	**Pond water**	**soil**
β-lactams	*bla*$_{SHV-82}$	Antibiotic inactivation	+	-	-
	bla$_{SHV-81}$		+	+	-
	bla$_{SHV-11}$		-	+	-
	bla$_{SHV-180}$		-	+	-
	bla$_{SHV-172}$		-	+	-
	bla$_{TEM-208}$		+	-	-
	bla$_{TEM-206}$		-	+	-
	bla$_{OXA-217}$		-	+	-
	bla$_{OXA-50}$		-	+	-
	bla$_{SED1}$		+	+	-
	bla$_{OKP-B-17}$		-	+	-
	bla$_{CMY-98}$		-	+	-
	bla$_{LEN-24}$		+	-	-
	*cfx*A2		+	-	-
	*cfx*A6		+	-	-
Tetracycline	*tet*(A)	Antibiotic efflux	+	+	-
	*tet*A(P)		+	+	-
	*tet*B(P)		+	+	-
	tet(D)		+	+	-
	tet(J)		+	+	+
	tet(L)		+	+	+
	*tet*O		+	-	-
	*tet*Q		+	-	-
	*tet*R		+	+	-
	tet(W)		+	-	-
	tet(W/N/W)		+	-	-
	*tet*X		+	-	-

Table 4.3.2: Acquired AMR genes from different sources

	tet(40)		+	-	-
Aminoglycoside	*aph* (6)-Id *aac* (6')-Iid *aac* (2')-Ia *aph* (3")-Ib *aph* (3)-IIb, *aac* (6')-Ii, *aac* (6')-Iz	Antibiotic inactivation	+ + + + - - -	+ + + + + + +	+ - - + - - -
Phenicol	*cat*III *cat*II *cat*B7	Antibiotic inactivation	+ - -	+ + +	- - -
Fluoroquinolone	*oqx*A *oqx*B	Antibiotic efflux	+ +	+ +	+ +
Macrolide	*mef*A	Antibiotic target protection	-	+	+
Fosfomycin	*fos*A *fos*A2 *fos*A3 *fos*A5 *fos*A6	Antibiotic inactivation	+ - - + +	+ + + + +	- - - - +
Trimethoprim-sulfamethoxazole	*dfr*A14 *dfr*A15	Antibiotic target replacement	+ -	+ +	+ -
Sulfonamide	*sul*1 *sul*2	Antibiotic target replacement	+ +	+ +	+ +

+: detected,
-: Not detected

Table 4.3.3: Chromosomal mutation AMR genes from different sources

AMR Class	**Gene**	**AMR Mechanism**	**Animal feces**	**Pond water**	**soil**
β-lactams	*bla*(*amp*H) *amp*C	Antibiotic inactivation	+ +	- +	- -
	*Omp*C *Omp*F *Omp*K35 *Omp*K36 *Omp*K37 *Omp*36 *Opr*D	Reduce permeability to antibiotic Resistance by absence(deletion)	+ + + + + - -	+ + + + + + +	- - + + + - -
Aminoglycoside	*acr*D cpxA	Antibiotic efflux pump	+ -	+ -	+ +
Phenicol	*CAT*	Antibiotic inactivation	+	+	+
Fluoroquinolone	*par*C *parE* *gyr*A *gyr*B	Antibiotic target alteration	+ + + +	+ + + +	+ - + -

Table 4.3.3: Chromosomal mutation AMR genes from different sources

	*emr*R	Antibiotic efflux pump	+	+	-
	*emr*A		+	+	+
	*emr*K		+	+	-
	*emr*E		+	+	-
	*emr*Y		+	+	-
	*emr*B		+	+	+
Fosfomycin	*Uhp*T	Antibiotic target alteration	+	+	+
	*Cya*A		+	+	-
	*Uhp*A		+	-	+
	*Glp*T		+	+	+
	*mur*A		+	+	+
	*Pts*I		-	+	+
Trimethoprim-sulfamethoxazole	*dfr*E	Antibiotic target replacement	+	+	-
	*dfr*F		+	-	-
	*dfr*G		-	+	-
Sulfonamide	*fol*P	Antibiotic target alteration	+	+	-
Multidrug class	*acr*A	Antibiotic efflux pump	+	+	+
	*acr*B		+	+	+
	acrE		+	+	-
	*acr*F		+	+	+
	*acr*R		+	+	+
	*acr*S		+	+	-
	*ade*K		+	+	-
	*ade*I		+	-	-
	*ade*B		+	+	-
	*ade*J		+	+	-
	*ade*F		+	+	-
	*ade*H		+	+	-
	*ade*G		+	+	-
	a*de*N		+	+	-
	*ade*L		-	+	-
	a*de*A		-	+	+
	*ade*I,		-	+	-
	*ade*S		-	+	-
	*ade*R		+	+	-
	*efr*A		+	-	-
	*efr*B		+	-	-
	CRP		+	+	-
	*mdf*A		-	-	+
	*nal*C		-	-	-
	*nal*D		-	+	-
	*ram*A		-	+	-
	*mex*P		-	+	-

+: detected
-: Not detected

4.4 Detection of plasmid genes

PlasmidFinder results revealed only a total of seven plasmid incompatibility groups (*Col*3M, *Col*RNAI, *Col*pVC, *Col* (MG828), *Col* (BS512), *Inc*FIB (K) and *Inc*R), which showed between 81.53 % and 100 % identity to query sequences. The *Col*3M, *Col*RNAI, and *Col*pVC plasmid genes were detected in both animal feces and pond water samples. Additional plasmids such as *Col* (MG828), *Col* (BS512), *Inc*FIB (K), *Inc*R) are shown in Table 4.4-1. Plasmid genes in soil and dog feces samples were not detected in the plasmidFinder database.

Table 4.4.1: Identification of plasmid genes from different sources

Plasmid Gene	Accession no.	Animal Feces	Pond water	Soil	Dog feces
*Col*3M	JX514065	+ (81.53 %)	+ (98.75 %)	-	-
*Col*RNAI	DQ298019	+ (99.23 %)	+ (84.62 %)	-	-
*Col*pVC	JX133088	+ (100 %)	+ (100 %)	-	-
Col (MG828)	NC008486	-	+ (98.47 %)	-	-
Col (BS512)	NC010656	-	+ (100 %)	-	-
*Inc*FIB(K)	JN233704	-	+ (100 %)	-	-
*Inc*R	DQ449578	-	+ (100 %)	-	-

+: (% coverage), detected
-: Not detected

4.5 Detection of virulence genes

Virulence factor genes were detected in animal feces and pond water, but not in soil and dog feces samples (Table 4.5.1). The virulence genes *iut*A, which encodes for ferric aerobactin receptor was identified in both animal feces and pond water. When comparing the attributes for virulence genes detected in animal feces and pond water, it was discovered that animal feces harbored the *gad* gene, which encodes for glutamate decarboxylase, whereas pond water samples contained genes such as Colicin ia (*cia*), various Tellurium ion resistance protein (*ter*C), ABC transporter protein (*mch*F), and the Outer membrane protein complement resistance membrane (*tra*T).

Table 4.5.1: Identification of virulence genes from different sources

Virulence Gene	**Protein function**	**Accession no.**	**Animal feces**	**Pond water**	**Soil**	**Dog feces**
gad	Glutamate decarboxylase	EF547380	+ (70.95 %)	-	-	-
*iut*A	Ferric aerobactin receptor	FLWH01000001	+ (63.01 %)	+ (100 %)	-	-
cia	Colicin ia	APXL01000039	-	+ (70.93 %)	-	-
*ter*C	Tellurium ion resistance protein	UGAE01000003	-	+ (100 %)	-	-
*mch*F	ABC transporter protein	AF063590	-	+ (81.88 %)	-	-
*tra*T	Outer membrane protein complement resistance membrane	FLWH01000012	-	+ (100 %)	-	-
*ter*C	Tellurium ion resistance protein	CP007491	-	+ (100 %)	-	-
*ter*C	Tellurium ion resistance protein	MG591698	-	+ (97.83 %)	-	

+: (% coverage) detected
-: Not detected

CHAPTER 5:DISCUSSION

5.1 Occurrence of ESBLs encoded viable and multi-drug resistant bacteria in animal feces, pond water and soil

Infections caused by ß -lactam-resistant organisms have surged in recent years because of the emergence of resistant ESBL enzymes and genes. Consequently, nearly all ß-lactam antibiotics are considered obsolete, posing a problem in clinical and veterinary settings (Aslam et al., 2018; Dhingra et al., 2020). This study demonstrates a baseline for detecting ESBL occurrences and other antibiotic resistance trends in potentially high-risk environments, all of which can help in the battle against AMR. In this study, different methods were explored to identify bacteria and classify antibiotic resistance determinants (specifically of β-lactamases and other antibiotic resistance genes) from cultured and uncultured samples using culture plating with different ß-lactam antibiotics and next generation sequencing methods, as well as bioinformatics tools to further identify AMR genes, MGEs (plasmids) and virulence genes within genomic and metagenomic DNA sequences.

This study suggests that antimicrobial resistant bacteria, particularly β-lactam resistance microbes, are present in the animal feces, pond water, and soil where animals and humans co-exist in the same household compound. This is of great concern to public health and this study has provided results in support of the high occurrence of antimicrobial resistance in different sources. The findings of this study were slightly higher but consistent with previous studies on the occurrence of ESBL encoded bacteria in various sources. A related study in Nigeria documented a similar high occurrence of ESBL encoded bacteria in farm workers and poultry, while the environment had fewer ESBL encoded bacteria than others (Aworh et al., 2020). In comparison to this study, which had higher levels of resistance to ampicillin (100 %) and cefotaxime (100 %) for animal feces, Carvalho et al. (2016), also found an

elevated level of resistance to ampicillin (85.7 %), but low level of resistance for cefotaxime (19.4 %), compared to this study.

For pond water, the resistance was consistent with numerous studies on non-hospital wastewater showing high resistance against β-lactam antibiotics (Rahman et al., 2021). Resistance to other antibiotics such as β-lactam, tetracycline, streptomycin, and trimethoprim-sulfamethoxazole were also detected. Studies in Germany (Falgenhauer et al., 2019), Korea (Lim et al., 2015), Nepal (Subramanya et al., 2021), Pakistan (Saleem et al., 2017) and the Netherlands (Leverstein-van Hall et al., 2011) have also demonstrated the occurrence of ESBL encoded antibiotic resistant bacteria (mainly *E. coli)* from different sources which included meat-producing livestock (such as poultry, cattle, and pig) and companion animals (such as dogs and cats), sewage and farm workers. From the findings of both this current study and others, due to the widespread use of β-lactams in clinical and veterinary settings, the β-lactam antibiotics are among the most widely detected class of antimicrobials with the increased risk of resistance transmission, as bacteria continues to advance by attaining resistance through different mechanisms such as production of β-lactamase enzymes, efflux pumps and target alteration of the binding sites (Worthington & Melander, 2013; Giani et al., 2017).

It is also notable in the present study that isolates from animal feces, pond water, and soil had a similar antimicrobial resistance pattern to the commonly used β-lactams (such as penicillin, ampicillin, cefoxitin, cefotaxime, and meropenem), which is suggestive that resistance spreads across different sources. A finding similar to this study was also recently demonstrated by Subbiah et al. (2020) revealing the similarity in the occurrence of different resistance phenotypes of antimicrobials (ampicillin, streptomycin, trimethoprim-sulfamethoxazole, and tetracycline) across different sources (people, animals, and the environment).

The similar resistance patterns of the bacteria from different sources in this study are also not surprising, and this trend appears to be prevalent in developing countries in Africa and Asia (Do et al., 2021) with poor communities due to a significant increase

in antimicrobial usage, poor handling, and misuse. The complex environment also provides a selective pressure for the antibiotic resistant bacteria evolution found in rural and peri-urban areas thus serving as hotspot for antibiotic resistant bacteria in the community (Berendonk et al., 2015; Durso & Cook, 2014; Founou et al., 2016; Williams-Nguyen et al., 2016).

5.2 Identification of bacteria and antibiotic resistance determinants in dog feces sample

In addition to culture methods, bacterial genome shotgun sequencing was utilized to provide comprehensive genomic data from a cultured sample of dog feces collected from Palapye household. This approach demonstrated the ability to identify several bacterial strains and ARGs in dog feces samples. From the findings of this study, the identified bacterial strains from cultured dog feces sample revealed clinically important bacterial species; *Proteus mirabilis*, *Citrobacter* sp. and *Paeniclostridium sordelli* (also known as *Clostridium sordelli)*. These bacterial strains are potentially pathogenic and could pose a major threat to humans by spreading infectious diseases such as urinary tract infections, gastrointestinal disorders, and sepsis in clinical settings (Armbruster et al., 2018).

Furthermore, the use of bacterial shotgun sequencing method revealed not only the bacterial strains present in the cultured dog sample but also the phylogenetic information indicating the relatedness of the predicted species and AMR genes present in the cultured dog sample. Some studies have indicated that dog feces harbor diverse microbes and this is influenced by the ecosystem in the gut (Garcia-Mazcorro & Minamoto, 2013; Deng & Swanson, 2014) . All these bacteria species can cause infections, mostly in humans with compromised immune systems and in host animals (Nandi et al., 2013;Nahar, 2014; Sultana et al., 2019).

Additionally, similar to the current study, there has been studies on domestic and companion animals describing the occurrence of different antibiotic resistant microbes (particularly ESBLs producing organisms) such as *Enterobacterales* (Hordijk et al., 2013;Carvalho et al., 2016; Yasugi et al., 2021), *Proteus mirabillis* spp. (Zappa et al., 2017; Han, 2019), *Citrobacter* spp. (Ewers et al., 2011) and *Clostridium sordelli* spp. (Capewell et al., 2020).

Many of the bacterial strains identified in this study can be found in water, soil, and the gastrointestinal tracts of both animals and humans. They are clinically important and thought to be pathogenic since they can cause clinical diseases such as nosocomial infections, urinary tract infections, enteric diseases, and gastrointestinal ailments (Samonis et al., 2008; Armbruster et al., 2018). *Proteus mirabillis* spp., for instance, is an opportunistic pathogen with virulence characteristics that make it more virulent. It is most linked to a hospital-acquired urinary tract infection, especially in patients with complicated catheters and weakened immune systems (Alatrash & Al-yasseen, 2017). Patients may develop cystitis, kidney stones, and bladder stones because of this bacterial strain (Nachammai et al., 2015).

Also, *Citrobacter* spp. has been identified as a major opportunistic pathogen that causes nosocomial urinary infections in hospitalized patients who are fragile and weak (Deal et al., 2007; Lavigne et al., 2007). The development of this multidrug-resistant pathogen has been rising rapidly, and considered one of the top pathogens that cause havoc in intensive care units especially in low- and middle-income countries and it has been demonstrated that it is resistant to routinely used antibiotics such as penicillins, cephalosporins, aminoglycosides, and fluoroquinolones (Ranjan & Ranjan, 2013). Finally, *Paeniclostridium sordellii* is a bacterial pathogen that has been linked to septic shock (associated with uterine infections) in women after natural or clinically induced termination, as well as gas gangrene in animals (Zane & Guarner, 2011; Capewell et al., 2020; Nyaoke et al., 2020).

Moreover, the bacterial strains reported in this study can easily spread through human-domestic animal contacts, resulting in clinical complications. Authors Subbiah et al.

(2020), Carvalho et al. (2016), Ljungquist et al. (2016) and Suchodolski (2011) proved this in their studies. Nonetheless, the extent to which resistant bacteria transmit between dogs and humans remains unknown. The findings of bacterial identification using different bioinformatics tools in our study also further emphasizes the value of bacterial genome sequencing in identifying various strains, genomic relationships, and source tracking, all of which are crucial for AMR encoded bacteria surveillance.

The bacterial strains detected in the dog feces sample also revealed a high occurrence of ARGs conferring resistance to phenicol, tetracycline, ß- lactam and multidrug classes. These ARGs were associated with different AMR mechanisms that included antibiotic efflux, target inactivation, target activation, target alteration, target protection, target replacement and target deletion. The predominant ARGs were associated with the target alteration and the multidrug efflux complex mechanisms. The multidrug resistance tripartite efflux resistance genes found in this study are known to confer multidrug resistance through overexpressed efflux pumps in several bacteria to various clinically important antibiotics (such as tetracycline, ß-lactams, chloramphenicol, ampicillin, nalidixic acid, and rifampin) hence imparting resistance by extruding antibiotics (Amaral et al., 2014; Blair et al., 2014). These multidrug efflux pumps found in dog feces bacteria confer multidrug resistance making common infections difficult to treat.

Several tetracycline resistance (*tet)* genes associated with efflux mechanisms were also discovered in this study which agreed with a study by Chang et al. (2015), who discovered *tet* genes in pets. Moreover, the genes resulting in target alteration mechanisms also have been found to be dominant in the dog feces sample. This mechanism occurs when there is chromosomal modification of the target site in order to block or reduce affinity of the target to antimicrobials (Argudín et al., 2017). The most common target modified genes discovered in dog feces were *gry*A and *gry*B chromosomal mutation genes conferring resistance to fluroquinolones. Comparably, these chromosomal mutation genes have been previously identified from dogs in other studies (Gibson et al., 2010; Shaheen et al., 2013; de Jong et al., 2018;; Umeda et al.,

2020). This was suggestive that careful usage of fluroquinolone antimicrobials in dogs and other domestic animals is necessary.

Surprisingly, this current study found an exceptionally low to no occurrence of β - lactamases genes in the dog feces, in contrast to some studies that found various variants of ESBL genes such as AmpC β-lactamase, *bla*$_{CMY}$, and *bla*$_{SHV}$, with *bla*$_{CTX-M}$ variants being the most prevalent across most studies in companion and domestic dogs (Carattoli et al., 2005; Pedersen et al., 2007 ; Zogg et al., 2018 Marchetti et al., 2021; Subramanya et al., 2021; Yasugi et al., 2021). Additionally, the findings of this current study were comparable with those of previous studies that identified an abundance of mutational multidrug resistance genes in domestic and companion animals (Sato et al., 2014; Arais et al., 2016; Umeda et al., 2020). These studies also suggest that resistance genes in animals pose a significant threat to public health, and the resistance mechanisms identified in this current study are important and may pose a risk of spread of multidrug ARGs to humans from animals through direct contact.

According to the current study findings, the ARGs encoded in the identified bacterial species and strains (*Proteus mirabilis* spp., *Citrobacter* sp. and *Paeniclostridium sordelli* sp.*)* could result in the spread of difficult to treat infections transferred between animals and humans, which would cause a huge problem in clinical and veterinary settings. Zogg et al. (2018) in Switzerland stated that domestic animals can play a critical role as reservoirs for ARGs, including ESBLs. Various ARGs conferring resistance to ß-lactam, tetracycline, and multidrug classes were discovered in dog feces isolates in some studies (Costa et al., 2008; Du et al., 2020). In comparison to other studies, our findings shed light on the occurrence and diversity of ARGs, as well as their mechanisms, in domestic dog feces. Furthermore, the discovery of these ARGs in the bacterial strains found in this study is concerning, dog feces should be considered an environmental pollutant of antibiotic resistant determinants, particularly in LMICs where there is poor sanitation.

5.3 Microbial community profiles revealing abundance and diversity from uncultured samples in animal feces, pond water and soil

The diversity and abundance of microbial community, ARGs, virulence genes, and plasmids in animal feces, pond water, and ambient soil were investigated using culture independent, high throughput shotgun metagenomics sequencing. The top dominant phyla across all samples were Proteobacteria (mostly Alpha-, Beta-, Delta-, and Gamma-proteobacteria), Actinobacteria, Firmicutes, Bacteroidetes, and Chordata. These findings are consistent with reports of the dominant microbial phyla found in ponds but mostly wastewater ponds (Vaz-Moreira et al., 2014; Qin et al., 2016; Chopyk et al., 2018; Jia et al., 2021) in animal feces (Salaheen et al., 2021) and in soil including sediments around the river, ponds, and wastewater (Parajuli et al., 2017; Espín et al., 2021) to be associated with mostly Proteobacteria, Firmicutes and Bacteroidetes. Notably, the phylum Bacteroidetes which is commonly detected in pond, feces and soil in this study was represented by low abundance of less than 7 %, which contrasts with other studies where the abundance is higher (Holman et al., 2017; Palanisamy et al., 2021).

According to these findings, the abundance of these phyla may be linked to their survival in highly contaminated and extreme environments (Selvarajan et al., 2021) and also, the different samples used in this current study are known to be natural habitats for diverse microbial community and the bacterial growth is influenced by these extreme environments which includes the poor sanitation and complex setup found in poor communities that are represented as “hot spots” of bacterial proliferation (Tamames et al., 2010).

The most abundant bacterial classes were associated with Gamma Proteobacteria across the different samples, with animal feces having the highest abundance, followed by pond water and environmental soil samples. This finding suggests that the high abundance of Gamma Proteobacteria in animal feces compared to other samples may be influenced by several factors which include the nutrient composition

in animal gut, their age, metabolic conditions, and other environmental conditions (Deng & Swanson, 2014). Notably, the Alpha Proteobacteria in soil was the second abundant class and the abundance was higher than in animal feces and pond water however, the abundance of Alpha Proteobacteria in soil from this study was consistent with a local study by Mhete et al. (2020) who revealed their findings to be in the range of 22 % to 32 % as compared to this current study's finding of 36 %. These findings suggests that numerous factors such as land use, soil type, physicochemical conditions, and climate change plays a role in altering the bacterial communities detected in soil samples and this was proven by Kuramae et al. (2011), Gazdag et al. (2018) and Mhete et al. (2020).

The two most abundant dominant genera at the genus level were *Klebsiella* and *Proteus*, followed by *Enterobacter* and *Escherichia*, which were the least abundant. They all belong to the class of Proteobacteria (Gamma Proteobacteria) and were quite similar across samples. Given the abundance of *Klebsiella pneumoniae, Proteus mirabilis,* and *Escherichia coli* species in this study, the results are not surprising as they are found to be abundant in soil, water, and feces samples. These genera are known to house opportunistic pathogens like *Klebsiella pneumoniae, Proteus mirabilis, and Escherichia coli,* all of which have the potential to infect humans and domestic animals (Drzewiecka, 2016; Moon et al., 2018; Henriot et al., 2019). This was consistent with findings of pathogens found in animal feces, in pond water and in soil that were linked to causing diseases such as nosocomial infections, urinary tract infections, diarrhea and food poisoning (Hordijk et al., 2013; Nandi et al., 2013; Zappa et al., 2017; Sultana et al., 2019; Tawyabur et al., 2020). From this study, it is evident that these species, especially *Klebsiella pneumoniae, Proteus mirabilis, and Escherichia coli,* also play a significant role in carrying ARGs and they are easily spread through feces contamination in water and soil samples, that can easily be contacted by humans. This was also proved by authors Pitout et al. (2005), Hasan et al. (2011) and Roca et al. (2015) on the presence of ARG-encoded microorganisms, mainly *Klebsiella pneumoniae* and *Escherichia coli* found in community and hospital settings.

5.4 Patterns of AMR genes and resistance mechanisms identified in animal feces, pond water, and soil

The findings of this study demonstrate that in a rural landscape, animal feces, pond water, and soil are crucial AMR hotspots with a high diversity and abundance of ARGs, including ESBL genes. This is in line with related studies that also determined the abundance of ARGs from different sources as associated with human, animal, and environmental samples in the community setup (Rousham et al., 2018; Cheng et al., 2020; He et al., 2020). From this study's findings, there is a notable observation of various ESBL genes. The most prevalent types across the literature are SHV and TEM (Paterson & Bonomo, 2005). However, some other ESBL types; OXA, OKP, CMY, LEN, CFX and SED were detected in pond water and animal feces samples (Bradford, 2001; Siebor et al., 2005; Fevre et al., 2005; Evans & Amyes, 2014;; San et al., 2020). In this study, the most detected ESBL genes were observed in pond water compared to other sources. This high occurrence of ESBL genes found in pond water can be influenced by domestic animal discharge and human activities associated with the use of pond water. The abundance of ARGs in water bodies has been reported to be highly influenced by different anthropogenic activities (Chen et al., 2010; Lu et al., 2010; Zurfluh et al., 2013; Li et al., 2015) , which is consistent with results from this study. Other previous studies have shown the CTX-M gene to be the most prevalent ESBL in animals and humans worldwide in various countries (Jouini et al., 2007; Sun et al., 2010; Tollentino et al., 2011; Maina et al., 2013; Osei Sekyere & Reta, 2020), which contrasts with the current study's findings, which found no CTX-M genes across all different sources.

The current study further revealed different gene variants within the *bla* $_{\text{SHV}}$ (*bla*$_{\text{SHV-82}}$, *bla*$_{\text{SHV-11,}}$ *bla*$_{\text{SHV-81}}$, *bla*$_{\text{SHV-180}}$ and *bla*$_{\text{SHV- 172}}$), *bla* $_{\text{TEM}}$ (*bla*$_{\text{TEM-208}}$, *bla*$_{\text{TEM-206}}$), *bla* $_{\text{OXA}}$ genes (*bla*$_{\text{OXA-217,}}$ *bla*$_{\text{OXA-50}}$), *cfx*A (*cfx*A2, *cfx*A6), *bla*$_{\text{LEN-24}}$, *bla*$_{\text{CMY-98}}$, *bla*$_{\text{SED1,}}$ and *bla*$_{\text{OKP-B-17}}$ were detected in animal feces and pond water. These different ESBL genes are all clinically important genes that are commonly responsible for difficult to treat infections in both human and veterinary settings (Costa et al., 2006; Ejaz et al., 2021). As a result of this diversity, the findings may suggest that animal feces and pond water

serve as a reservoir of ESBLs among other ARGs and can be disseminated to other hosts and the environment. These findings are slightly in line with studies from Nigeria (Awosile et al., 2020), which reported the presence of *bla*$_{\text{SHV-11,}}$ *bla*$_{\text{SHV-12}}$, and *bla*$_{\text{CMY}}$ in both animals, humans, and environmental sources. In Italy, Carattoli et al. (2005) also documented the *bla*$_{\textit{CMY}\text{-2,}}$ *bla*$_{\text{SHV-12}}$, and *bla*$_{\text{CTX-M-1}}$ from domesticated dogs and cats. The current study identified most of the *bla* genes from pond water which is in parallel with a study by Vivas et al. (2019) on ESBL genes (*bla*$_{\text{TEM-1}}$, *bla*$_{\text{CTX-M 9}}$, *bla*$_{\text{VIM-2}}$, *bla*$_{\text{IMP-1}}$, and *bla*$_{\text{AmpC}}$) in water sources. Both these findings suggest that the high occurrence of *bla* genes in pond water, and other water environments is influenced by the selective antibiotic pressures, which contribute to further evolution of ARGs encoded in diverse microbial communities found in these sources (Bréchet et al., 2014). Furthermore, the detection of *cfx*A genes in this study that were found in animal feces are commonly associated with anaerobe microbes and is consistent with studies from (Skarżyńska et al., 2020; Suriyaphol et al., 2021).

Additional to the ESBL genes revealed in this current study, chromosomal mutation genes *(Omp*K35, *Omp*K36, *Omp*K37) associated with alteration of outer membrane porins in *Klebsiella pneumoniae* were detected across all different samples. These mutant genes interfere with the outer membrane porins of the bacterial cell thus making the entrance of the β-lactam antibiotics impossible. Doménech-Sánchez et al. (2003), Tsai et al. (2011), Chi et al. (2019) and Kim et al. (2020) corroborated with these current study's findings demonstrating the occurrence of these point mutation genes associated with ESBL-producing *K. pneumoniae*. The findings of this current study are also similar to previous studies from Ecuador (Salinas et al., 2021), Germany (Pietsch et al., 2018; Herrig et al., 2020; Savin et al., 2020), and Kenya (Maina et al., 2013; Hassell et al., 2019; Saisi et al., 2019), further reinforcing the need to adopt the one health approach in tracking clinically important ARGs such as ESBL genes. This study provides further proof of the dynamic environments and their role in the dissemination of AMR determinants, which is supported by diverse ARGs patterns in rural environments and poor communities associated with poor sanitation.

To further determine the threat of AMR in Palapye community households, this current study investigated other ARGs and their mechanisms that may play a role in resistance patterns in the environment (soil and water) and domestic animals. Resistance genes to clinically important antimicrobial classes; tetracycline, phenicol, aminoglycoside, fluoroquinolone, macrolide, fosfomycin, trimethoprim-sulfamethoxazole, and sulfonamide were detected. These findings were consistent with previous studies indicating the co-occurrence of other ARGs with ESBL genes possibly connecting the co-existence of antibiotic selection pressure which in turn might influence the dynamics of antibiotic resistance in the different sources (Börjesson et al., 2009; Ju et al., 2016; Seni et al., 2016; Khan et al., 2020). In addition, chromosomal point mutations of multidrug resistance genes linked to several mechanism which included antibiotic efflux, antibiotic modification, and antibiotic replacement/deletion were also discovered across the different sources in this study which add to the co-selection of the ARGs in the interconnected environments.

In this study, pond water revealed the most prevalent ARG mechanisms conferring resistance to β-lactams, aminoglycoside and multidrug mutational resistance genes compared to animal feces which revealed resistance patterns amongst classes of tetracyclines, β-lactams and multidrug mutational resistance genes. This study indicates the presence of diverse ARGs patterns in a single rural household. The findings showed that pond water had more different ARG genes, followed by animal feces and lastly soil samples. Similar to the current study, a previous study on ARGs in pond water done by Finton et al. (2020) demonstrated pond water to have elevated levels of ARGs, including those associated with MDR Efflux pumps that have resistance to multiple classes of antibiotics (*acr*AB, *emr*AB, *mdt*H, *mdf*A), as well as resistance to tetracycline (*tet*(A), *te*t(B)), macrolide (*mac*A, *mph*A), fluoroquinolones (*oqxA, oqxB),* trimethoprim (*dfrA*), fosfomycin (*fosA),* aminoglycosides *(aac* (3)-I) and sulfonamides (*sul1*).

Furthermore, mutations in *gyr*A, *gyr*B, *par*C, and *par*E were found in pond water in the current study, which correlates with a studies by authors (Adachi et al., 2013; Zurfluh et al., 2014; Johnning et al., 2015) that found an abundance of mutational

fluoroquinolone resistance in aquatic environmental microbial community. These findings suggest the occurrence of multiple drug-resistant bacteria and acquired and chromosomal ARGs in surface water that is likely to be used for a variety of purposes, including human (children playing in the ponds and using pond water for construction) and animal activities. The presence of these ARGs in the environment poses a serious health risk to humans and animals in the surrounding, while also demonstrating the relevance of the water linked environment as a hub for this rising problem of fluoroquinolone resistance.

The abundance of clinically relevant ARGs found in animal feces samples in the current study were associated with tetracycline (*tet* (W), *tet* (40), *tet*(A), *tet*(J), *tet*Q, *tet*A (P), *tet*R, *tet*(W/N/W), *tet*X, *tet* (L), *tet*O, *tet*B(P), *tet*(D)) which indicated to be the most prevalent ARGs compared to other antibiotic classes. Similarly, a study by Bryan et al. (2004) discovered tetracycline genes (*tet*B, *tet*A, *tet*C, *tet*D and *tet*M) in animals feces, confirming its abundance in the United States of America. These findings are expected, given that tetracycline is widely used in human and animal maintenance care, and that most used antibiotics in animal maintenance are easily accessible over the counter in countries such as South Africa, Ghana, and Rwanda (van Boeckel et al., 2015; Boamah et al., 2016; Manishimwe et al., 2017 Jaja et al., 2020), as is the case in Botswana. Other previous studies agree with these current findings on the detection of tetracycline in animals and environmental sources (Cheng et al., 2013; Wang et al., 2019; Gu et al., 2020; Hartinger et al., 2021) . These findings provide insight into the real impact of animal feces as contaminants of ARGs as well as contaminated pond water and soil. These are drivers of ARGs to the immunocompromised adults and young children in poor communities.

Other ARGs found in animal feces in this current study included aminoglycosides genes, phenicol, fluoroquinolone, fosfomycin, trimethoprim-sulfamethoxazole, and Sulfonamide which correlated with findings from previous studies, undertaken by authors (Hammerum & Heuer, 2009; Veldman et al., 2011; Szmolka & Nagy, 2013; Alonso et al., 2017) . These findings together with the current study, highlight the critical need for more monitoring and management in the use of veterinary

medications in order to limit an alarming rise of antimicrobial resistance in the country.

In the current study, ARGs in soil samples were fewer than animal feces and pond water, even though soil is known to be the largest reservoir for ARGs as they occur naturally in soils. Nevertheless, various ARGs including tetracycline, aminoglycosides, sulfonamides, and multidrug mutational resistance genes were detected in the environmental soil of the household. Similarly, a study by (Qian et al., 2021) demonstrated the presence of multidrug resistance genes, tetracycline (*tet*A, *tet*C, *tet*M, *tet*O, *tet*V, *tet*X) and aminoglycoside *(aac*(*2'*)-*I*, *aac*(*6'*)-*I*, and *aph*(*6'*)-*I*) that confer resistance to commonly used antibiotics in common infections affecting human and animals. Moreover, numerous studies have indicated that high abundance of ARGs in soils can also be influenced by anthropogenic activities that include agricultural, human, and animal activities (Hu et al., 2010 ; Wang et al., 2014 ; Wu et al., 2017 ; Tian et al., 2021). These findings demonstrate that not only does ARGs come naturally from the soil, but different activities and soil use can influence the abundance of clinically relevant ARGs which pose a threat to the public as they can easily be spread to humans and animals.

In relation to this study, closely similar studies using one health approach were done by (Purohit et al., 2017; Hartinger et al., 2021) who showed a high occurrence of antibiotic resistance genes and co-resistance patterns in humans, environment (soil and water) and animal samples. These studies agreed with the current study that AMR is understudied in rural communities, despite the high interconnectivity of animal, human, and environmental sources in an inadequate sanitation condition. The poor communities and poor sanitation found in LMICs promotes an extremely high selective pressure environment for different antibiotic resistant bacteria to confer resistance to multiple antibiotics hence posing serious public health risk. Other studies connected to the current study findings suggested that surface water, animal and human feces could be potential hosts for resistant bacteria in the environment, which could be important for the spread of ARGs (Baquero et al., 2008; Molton et al., 2013;

Rolain, 2013; Allen, 2014; Pruden, 2014; Bengtsson-Palme et al., 2015; McEachran et al., 2015; van Schaik, 2015).

5.5 Identification of MGEs and virulence genes from different sources using bioinformatics tools

Next generation sequencing provides more data that is also relevant to this study. From the metagenomic data, this study was also able to reveal mobile genetic elements and virulence genes, which complement the AMR data. The use of bioinformatics tools like plasmidFinder and virulenceFinder has made plasmid replicon and virulence factor prediction in shotgun metagenomics sequences much easier. This current study explored these bioinformatics methods to understand more about the occurrence and diversity of MGEs such as plasmids. Plasmids play a crucial role in the transmission of ARGs especially the ESBLs and they are critical in the spread of ARGs which mostly occur via horizontal gene transfer mechanism among bacterial species.

In this study, the plasmid incompatibility groups that were detected included *Col*3M, *Col*RNAI, *Col*pVC, *Col* (MG828), *Col* (BS512), *Inc*FIB (K) and *Inc*R in both animal feces and pond water. These plasmid incompatibility replicons have been linked to the spread of important ARGs, such as ESBLs that confer extended spectrum resistance to β-lactams, in clinical *Enterobacterales* such as *Klebsiella pneumoniae* (Carattoli, et al., 2014; Roosaare et al., 2018). Some studies discussed further these various plasmid groups and their potential in mobilizing ARGs in different environments (Ma et al., 2012; Zurfluh et al., 2013; Kudirkiene et al., 2018; Founou et al., 2019; Patil et al., 2019; Salinas et al., 2021). Bitar et al. (2020) also discovered a plasmid replicon (pIB *COL*3M) containing the quinolone resistance gene (*qnr*D1), while Ogbolu et al. (2013) reported that *Inc*F plasmids are drivers encoding for ß-lactamases. Similarly Yang et al. (2015) and Coque et al. (2008) demonstrated that the *Inc*FII plasmids are known as the prevalent plasmids that house the MDR genes.

Furthermore, several virulence genes known to play key role in bacterial pathogenesis were discovered in animal feces and pond water. The most prevalent virulence factor identified in both sources included *gad* and *iut*A genes. The *gad* (glutamate decarboxylase) gene is associated with bacterial survival in extreme acidic environments (animal guts) by regulating the intracellular pH (Chen et al., 2018) while *iut*A genes encodes for Ferric aerobactin receptor (Malekzadegan et al., 2018) which also plays a role in mediating the uptake of ferric iron. This influences the adaptation of the virulent bacteria to the new niche thus allowing them to increase their capacity to cause a wider range of diseases. *P. mirabilis*, for example, employs a variety of virulence factors to gain access to and invade the human urinary tract hence causing urinary tract infections (Schaffer & Pearson, 2015).

Other virulence associated genes included colicin gene (*cia),* tellurium ion resistance gene (*ter*C), ABC transporter protein gene (*mch*F) and outer membrane protein gene *(tra*T) were detected, these virulence factors are mainly associated with transfer protein, and uptake of iron (Sarowska et al., 2019). None of the most clinically challenging enterotoxin virulence genes were discovered in our study. The identified virulence genes, on the other hand, could still play a role in environmental hosts of the bacterial community, as they could increase adaptation and resilience of pathogenic ESBL encoded bacteria found in animal feces and pond water that could eventually become human pathogens. These findings further imply that the presence of both ARGs and virulence factors in the bacterial community constitutes a significant threat to clinical therapy of common illnesses.

CONCLUSION

The current study findings have proven the hypothesis, there was a relatively high occurrence, diversity and abundance of bacteria, particularly ESBL encoding, and highly resistant bacteria identified from both cultured and non-cultured samples of animal feces, pond water, and soil obtained from a rural community in Palapye. It is evident that the complex environment depicted by Palapye household provides a dynamic, high selective pressure environment for the antibiotic resistant bacteria evolution and dissemination of ARGs. This is highly influenced by the rural environment, which is associated with poor sanitation, the rearing of livestock around households, and the storage of water in unsanitary conditions. The study further provided proof that there is a high abundance of resistance genes, including ESBLs, in domestic animals kept in households, thus posing a significant threat to public health as there is a risk of the spread of multidrug ARGs to humans through direct contact resulting in clinical complications.

This also highlights the critical need for increased monitoring and management of veterinary and clinical medications in order to limit the country's alarming rise in antimicrobial resistance. The surface water used by animals and people in the household also represented an important pool of resistance genes that conferred resistance to clinically relevant antibiotics, thus limiting treatment options. According to this study’s findings, the use of one health approach has also provided an insight into the dissemination and spread of ARGs in different sources as well as the use of different bioinformatics tools that further emphasized the value of microbial identification, genomic relationships, and source tracking, all of which are crucial for AMR encoded bacteria surveillance.

Nevertheless, it is noteworthy that this pilot study has set a baseline for detecting ESBLs and other antibiotic resistance patterns in a potentially high-risk community setting. The findings of this study will be used to influence future research on the state of antibiotic resistance patterns and current ESBL dynamics, with the study being expanded to include more rural communities in Botswana directly linked to domestic animals, fields irrigated with wastewater, and feces obtained from young children.

Even though this study's findings may not be generalized as the inclusive situation of the country, it is significant as it highlights a clear indication that there is a high prevalence of clinically important ESBL producing bacteria in Palapye and the domestic animals and the environment have been gradually shown to be play a significant role in the dissemination of ARGs among all aspects of the community. Therefore, it is necessary for clinicians, veterinarians, farmers, and the community to be made fully aware of the ESBL producing microorganisms as well as the proper usage of ß-lactam antimicrobials.

RECOMMENDATIONS

The findings from this study will assist government health agencies, which will utilize the information to educate the public about the probable routes of antibiotic resistance transmission and how they may protect themselves in rural communities. Furthermore, these data will be useful both locally and internationally for comparison of AMR trends, improvement of AMR surveillance systems, and can be used to drive initiatives on antibiotic stewardship, such as Botswana's National Action Plan (NAP) on antibiotic resistance.

Furthermore, the following areas can be revised, and future research can be implemented.

- Implementation plans on antibiotic prescription in rural community settings and education on appropriate dispensation of the most recent and critically important β-lactam antibiotics to prevent resistance development.
- More public education on the proper use and misuse of antibiotics, which leads to the growth in antibiotic resistance and community mortality, should be provided by the relevant stakeholders.
- Future studies should have all-inclusive data from different sources, including clinical and environmental isolates, that will clearly depict the "One Health" approach to adequately evaluate the ARGs and MGEs transmission routes across all different disciplines.
- In future, a survey may be conducted to check what antibiotics are mostly used and how frequently they use them in both humans and animals to make the work more interesting.

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APPENDIX

Bacterial species identified in dog feces sample using KmerFinder

Predicted species	Query coverage (%)	Template coverage (%)	Accession no
Proteus mirabilis strain CRPM10 chromosome, complete genome	44.31	81.77	NZ_CP043332.1
Citrobacter sp. RHB21-C05 chromosome, complete genome	6.34	12.69	NZ_CP057611.1
Paeniclostridium sordellii strain AM370 chromosome, complete genome	2.24	4.93	NZ_CP014150.1
Proteus mirabilis strain AR_0059, complete genome	1.89	3.48	NZ_CP020052.1
Proteus mirabilis Strain PmSC1111 chromosome, complete genome	0.69	1.29	NZ_CP034090.1

The Kmerfinder method was used to predict bacteria organisms present in the dog feces sample. Five top hits predicted species and strains included; *Proteus mirabilis strain CRPM10 chromosome, complete genome* species with query coverage of 44.31 %, *Citrobacter sp. RHB21-C05 chromosome, complete genome* with query coverage 6.34 %, followed by *Paeniclostridium sordellii strain AM370 chromosome, complete genome* with query coverage 2.24 % and finally *Proteus mirabilis strain AR_0059, complete genome* and *Proteus mirabilis strain PmSC1111 chromosome, complete genome* with query coverage 1.89 % and 0.69 % respectively.