DETECTION OF ANTIBIOTIC RESISTANT *ESCHERICHIA COLI* IN CHILDREN LIVING IN A RURAL COMMUNITY OF LWAMONDO VILLAGE IN LIMPOPO PROVINCE AND THEIR ENVIRONMENT

By

MPHEGO MPHO

(17003211)

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UNIVERSITY OF VENDA

THOHOYANDOU

SOUTH AFRICA

SUPERVISOR: DR SE LEDWABA [Rhodes University]

CO-SUPERVISOR: PROF N POTGIETER [Univen]

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DECLARATION

I, Mpho Mphego (Student number: 17003211) declare that this dissertation for the award of master's degree in microbiology (MNMMMS MSc MBY) at the University of Venda is my original work and has not been previously submitted for any degree at any other University or Institution. All reference materials contained herein have been duly acknowledged.

Signed  **Date** 03/02/2025

DEDICATION

I dedicate this dissertation to the Almighty God for His protection and guidance throughout my academic life. I also dedicate this work to my family for their motivation, encouragement, and moral support.

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ABSTRACT

Background: *Escherichia coli (E. coli)* is found almost in every environment and in the human body. Human-animal-environment interactions may be driving the spread of antibiotic resistant bacteria, particularly in areas with little restrictions on antibiotic use, widespread food animal production, and free-roaming domestic animals. Children who are exposed to domestic animals and their waste in the home environment are more likely to have intestinal colonisation of antibiotic-resistant bacteria. The main aim of this study was to detect the antibiotic resistance patterns of *E. coli* isolates from children less than 5 years old and from their environment.

Methods: In this study, a total of 94 samples were collected from children (47 stool samples) and the environment (47 soil samples, in each household where the stool was obtained). Isolation of *E. coli* was done using standard culture methods on Eosin methylene blue and MacConkey agars. The Kirby Bauer disk diffusion method was used to determine the antibiotic resistance of *E. coli* isolates. The DNA of all *E. coli* isolates was extracted using the boiling method following standard protocols and confirmation of the presence of *E. coli* and determination of resistant genes was done using PCR. The *E. coli* isolates were then further tested for phylogenetic grouping using PCR employing three specific genes, namely *TspE4c2, yjaA.1* and *chuA.*

Results: The results showed that 216 isolates (117 from stools and 99 from soil samples) were presumptively identified as *E. coli*. A total of 211 isolates were confirmed as *E. coli* using PCR. Antibiotic resistance testing showed high resistance to chloramphenicol in soil (50%) and stool samples (41%). A total of 33% (70/94) isolates were positive for bla_{TEM} gene in both soil and stool samples. Phylogroup A was predominant [87%; 65/75] followed by phylogroup D [13%; 10/75], and phylogroup B [1%; 1/75].

Conclusion: The soil environment plays an important role in the transmission of antibiotic resistant *E. coli* in young children. It is critical to emphasize the need of adhering to proper hygiene standards and the appropriate use of antibiotics.

Keywords: Antibiotic resistance, children, diarrhoea, *E. coli*, Kirby Bauer disk diffusion, PCR.

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LIST OF ABBREVIATIONS

ABBREVIATION	DESCRIPTION
%	Percentage
bp	Base pairs
˚C	Degree Celsius
ml	millilitres
V	Volts
UV	Ultraviolet
MIC	Minimal inhibitor concentration
MDR	Multi-drug resistance
AGRISAR	Advisory group on intergrated surveillance of antimicrobial resistance
AR	Antibiotic resistance
ARB	Antibiotic resistance bacteria
ARGS	Antibiotic resistance genes
AMR	Antimicrobial resistance
bla$_{TEM}$	Beta-lactamase gene *TEM*
bla$_{CTX-M}$	Beta-lactamase gene *CTX-M*
bla$_{OXA}$	Beta-lactamase gene *OXA*
CLSI	Clinical and Laboratory Standard Institute
DNA	Deoxyribonuclease acid
Cfu	Colony-forming unit
DAEC	Diffusely adherent *Escherichia coli*
DEC	Diarrhoeagenic *Escherichia coli*
E. coli	*Escherichia coli*
ESBLS	Extended spectrum β-lactamases
EAEC	Enteroaggregative *Escherichia coli*

EHEC	Enterohaemorrhagic *Escherichia coli*
EIEC	Entero-invasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
F	Forward
HGT	Horizontal gene transfer
HIV	Human Immuno-deficiency virus
LT	Heat-liable
M	Meters
PCR	Polymerase Chain reaction
PBS	Penicillin binding proteins
PBS	Phosphate buffer saline
R	Reverse
Spp.	Species
STATSSA	Statistics South Africa
Stx	Shiga toxin-producing *Escherichia coli*
TSB	Trytone soya broth
TSI	Triple iron sugar
UN	United Nations
WHO	World health organisation
WASH	Water, sanitation, and hygiene

Chapter 1
GENERAL INTRODUCTION

1.1 INTRODUCTION

Escherichia coli is a widely recognised pathogen due to its ability to adjust to, colonise, and survive in a variety of environments through a number of acquired virulence factors (Mueller and Tainter, 2023). Its presence has an impact on the food, agriculture, and health sectors (Oliveira et al., 2023). Globally, 1.7 billion diarrhoeal infections occur annually, resulting in 525,000 deaths among children under the age of 5 (World Health Organisation, 2017; Aba et al., 2023). Inadequate water sources, unsanitary living conditions, and poverty all contribute to the diarrhoea that children experience (Ingle et al., 2018).

Children's intestinal microbiomes develop in the first three years of life after acquiring bacteria from their mothers during pregnancy (DeFrancesco et al., 2017). Their immature immune systems expose them to harmful organisms, increasing their susceptibility to diseases (Gollwitzer and Marsland, 2015). Malnutrition in low-resource settings increases susceptibility to infections (Ibrahim et al., 2017). Although it is unclear whether malnutrition causes more infections or exacerbates disease severity, malnourished children are more likely to die (Rytter et al., 2014).

Antibiotics are drugs used to treat human infections caused by bacteria, either by killing or inhibiting their growth and proliferation (Ardal et al., 2020). The production of ethanol and anti-fouling paints, as well as domestic use, are among numerous uses of antibiotics in aquaculture, horticulture, livestock husbandry, bee-keeping, and fish farming (Meek et al., 2015; Van et al., 2020; Miller et al., 2022). Antibiotics that have a β-lactam ring are known as "*β-lactam antibiotics*" (Zango et al., 2019). Cephalosporins, monobactams, carbapenems and penicillins are examples of antibiotics categorised as β-lactams (Holten and Onusko, 2000; Silva, 2022). By producing an enzyme called β-lactamase, which targets the β-lactam ring, bacteria can become resistant to β-lactam antibiotics (Pandey and Cascella, 2019).

As stated by the World Health Organisation, antibiotic resistance is a natural phenomenon, causing increased costs for healthcare providers, testing, hospital

stays, and even premature death (WHO, 2020). The total cost of antibiotic resistance to society is difficult to determine due to several factors, including the need for expensive second-line drugs, longer hospital stays, and increased morbidity (Bergeron et al., 2015).

In developing countries, household surfaces and soil are significant sources of transmission for diarrhoeal illnesses (Navab-Daneshmand et al., 2018). These environmental compartments are linked to enteric bacterial spread, with individuals being more susceptible to illness variations due to early colonization (DeFrancesco et al., 2017). Humans constantly interact with soil, potentially serving as vectors and intermediaries for *E. coli* antibiotic resistant and pathogenic strains (Santamaría and Toranzos, 2003; Montealegre et al., 2018). Commensal bacteria from intestinal microbes, such as coliforms, *E. coli, Clostridium difficile*, and *Enterococcus faecium*, have an important influence on the dissemination of antibiotic resistance in humans. (Gasparrini et al., 2019; Tougas et al., 2021).

Previous studies indicate that water and soil from outdoor playgrounds are frequently infected with different pathogens at the same time, suggesting that children who ingest soil could be consuming doses of various pathogens (Baker et al., 2018; Medgyesi et al., 2019). *E. coli* and humans co-exist in a mutualistic relationship (Shkoporov et al., 2022). *E. coli* is a typical bacteria located in the guts of warm-blooded animals and human, and helps to digest food, produce vitamins K and B, and prevent harmful bacteria from colonising the intestine (Martinson and Walk, 2020). *E. coli* can spread from an infected host's faeces to another host via the faecal-oral pathway, which is facilitated by environmental reservoirs. Furthermore, as children under the age of 5 learn to crawl, these individuals could pick up contaminants or other contaminated objects and put them in their mouth (Workie et al., 2019). Contact with contaminated water (Dos Santos et al., 2023), soil (Aditya et al., 2023), hands (Shaffer and Lozupone, 2018; Parvez et al., 2019; Cantrell et al., 2023), flies (Monyama et al., 2022; Nayduch et al., 2023), and food (Luna-Guevara et al., 2019) can all contribute to transmission. Playground soils are often contaminated with parasites, viruses, and bacteria, among other infectious agents (Chatziprodromidou et al., 2022). These agents have the ability to survive, complete their life cycles, and persist in the area until they are consumed (Chatziprodromidou et al., 2022). Soil in rural villages such

as the Lwamondo village in South Africa was previously discovered to be highly contaminated with *E. coli* and *Serretia marcescens,* implying that soil could be a source of contamination (published dissertation). Children that play, walk without shoes, ingest soil, or fail to perform basic personal hygiene are at greater risk of encountering contaminated soil (Hailegebriel et al., 2020). In peri-urban Harare, Zimbabwe, households with children had substantial amounts of *E. coli* contamination in drinking and handwashing water, soil, and hands (Navab-Daneshmand et al., 2018). Ledwaba et al (2019) investigated the transmission of diarrhoeagenic *E. coli* in toys used by children in the district of Vhembe and found a significant amount of *E. coli* contamination.

The increasing usage of β-lactam antibiotics for treating bacterial infections has led to a global health concern over antibiotic resistance (Pandey and Cascella, 2019). The synthesis of β-lactamases is a significant and dangerous form of resistance (Lin et al., 2015). β-lactamase inactivates drugs by hydrolysing their β-lactam ring which leads to treatment failures (Eiamphungporn et al., 2018). In the South African province of Limpopo, an investigation was done on the patterns of antibiotic resistance and the detection of β-lactamase in *E. coli* obtained from young children who had diarrhoea, the outcomes showed that both children who had diarrhoea had previously been exposed to antibiotics, and those who had not showed antibiotic resistance had the same strain of *E. coli* (DeFrancesco et al., 2017). In *E. coli* from children under five years old, the frequency of bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, and co-occurence of β-lactam genes were confirmed by a study conducted in Ethiopia (Zenebe et al., 2023). A high prevalence of diarrhoeagenic *E. coli* (DEC) antimicrobial resistance to antibiotics such as ampicillin (98%), tetracycline (91%), chloramphenicol (94%), and trimethoprim sulfamethoxazole (96%) was detected in a study on the antibiogram pattern and prevalence of pathotypes associated with diarrhoea cases in children in the Amatole District Municipality of Eastern Cape, South Africa (Omolajaiye et al., 2020). Antibiotic resistance rates among *E. coli* isolates in children who were healthy in India was higher for ampicillin (100%), nalidixic acid (100%), and cefotaxime (99.2%) (Purohit et al., 2019). The most frequently administered antibiotics used to treat diarrhoea, such as ampicillin, clarithromycin, and erythromycin, have a higher resistance rate to diarrhoeagenic *E. coli* (DEC), and gentamycin had the lowest resistance to DEC,

according to a study conducted in North West province, South Africa, that investigated the antibacterial resistance profiles of *E. coli* strains obtained from children under the age of five as well as drinking water and the clonality of the isolates (Chukwu et al., 2019). As a result, the high prevalence of DEC resistance to these routinely used antibiotics could be a serious threat to public health (Chukwu et al., 2019).

1.2 STUDY RATIONALE

In the Vhembe district, little research has been reported on the identification of *E. coli* that is resistant to antibiotics used in children. Most households in Lwamondo village engage in subsistence farming activities, such as cultivating crops and rearing domestic animals. Children in these homes frequently interact with the environment and animals, exposing them to trace amounts of bacteria in their natural environments, especially in soil (Schachter et al., 2020). This exposure, particularly in community settings, increases the chances of carrying antibiotic-resistant commensal bacteria and these actions may contribute to the growth or spread of bacteria resistant to antibiotics (Singh et al., 2018). Therefore, the study is aimed at evaluating antibiotic resistant *E. coli* in children and their environment.

1.3 PROBLEM STATEMENT

Due to poor living conditions in Lwamondo village, children under 5 years old are at higher chances of attaining antibiotic resistant *E. coli*. Community members participate in different agricultural activities such as farming and rearing of animals in their homes. These activities can result in the development of bacteria resistant antibiotics and possible transmission route between mother and child; and other environmental reservoirs (soil). Children younger than five years old could be at risk from environmental pathogenic strains that can cause diarrhoeal illnesses.

1.4 STUDY OBJECTIVES

1.4.1 MAIN OBJECTIVE

- To detect the antibiotic resistance of *E. coli* in children less than 5 years old and their environment.

1.4.2 SPECIFIC OBJECTIVES

- To isolate *E. coli* in children's stool samples and associated environmental (soil) samples.

- To determine the antibiotic susceptibility profiles of *E. coli* isolates using disk diffusion method.
- To confirm the isolated *E. coli* using PCR.
- To detect antibiotic resistance genes of *E. coli* using published PCR protocol.
- To determine the phylogroups of antibiotic resistance genes of *E. coli.*

Chapter 2

LITERATURE REVIEW

2.1 BACKGROUND

Diarrhoeal episodes affect children in developing nations with poor sanitation on average four to eight times a year between the ages of one and two, demonstrating constant exposure to enteric pathogens from an early age (Kotloff et al., 2013). Approximately 14 million South Africans, 2.5 million of whom reside in Limpopo and 0.6 million in the Vhembe area, lacked access to proper sanitation in 2013 (Poague et al., 2022). Children may be given antibiotics even though they do not need them because many paediatric infections would have healed without them (Medernach and Logan, 2018). Antibiotics are known to be prescribed to children more frequently than any other form of drug due to their recurrent infections of various aetiologies (Medernach and Logan, 2018). Overuse of antibiotics can lead to bacterial mutation and resistant development (Romandini et al., 2021).

In developing countries, faecal contamination of soil and surfaces in homes is a significant source of diarrhoeal disease exposure (Holcomb et al., 2020). Soil may be contaminated by both animal and human activity (Rodrigues and Römkens, 2018). Open defaecation, improper disposal of infant and children's faeces, and poor handling of animal faeces are likely to be factors in soil faecal pollution (Ngure et al., 2013).

2.2 SANITATION AND HYGIENE CONDITIONS IN RURAL COMMUNITIES

According to the most recent data, 60% of people or 4.5 billion, lack access to sanitary facilities that are safely managed (World Health Organization, 2018). Environmental factors including the shortage of clean water and proper sanitation usually have an impact on the health of adults over 50 years old and children, especially those under the age of five (Omotayo et al., 2021). According to Rogowski McQuade et al (2020), reducing children's exposure to pathogens by good hygiene practices, clean water, and sanitation can help prevent diarrhoeal infections in children under five, especially in low-resource settings.

Sanitation, and hygiene are all linked to diarrhoea and malnutrition in many ways for example, exposure to faeces through inadequate sanitation, and poor hygiene causes diarrhoea (Ngure et al., 2014). Studies shows that maintaining proper hand washing

before feeding children is crucial for preventing diarrhoea and other illnesses (Deshmukh et al., 2009; Mohammed and Tamiru, 2014). In sub-Saharan Africa, 215 million people or 35% of the rural and 8% of the urban populations are reported to still excrete in the open (Galan et al., 2013). Therefore, one of the most common causes of mortality among children below the age of 5 years in Sub-Saharan Africa, diarrhoeal infections are facilitated by this practice (Ayalew et al., 2018). According to Nguyen et al. (2021) in Cape Town, South Africa, there is a significant association between diarrhoea and the following factors: applying pesticides inside the house, using the same sanitation facility with more than four households, having an unsafe toilet, and poor hygiene practices in children. A high frequency of open defaecation (59%) and unsanitary practices (78%) in close proximity to water sources, lack of source water protection, and source proximity to pit toilets were all strongly correlated with a significant frequency of *E. coli* that was identified in drinking water samples (Gwimbi et al., 2019). Ledwaba et al (2019) revealed that children defaecate in open spaces in the yard in day care centres (50%) and used a children's toilet (35%) in rural households in the Vhembe district, Limpopo, and these behaviours increase the prevalence of *E. coli* infections in children.

2.3 Escherichia coli

The Enterobacteriaceae family of bacteria includes the Gram-negative *E. coli*, which has short bacilli (**Figure 2.1**) of nearly 0.5 µm in diameter and between 1-3 *µ*m in length (Singleton, 1999). It is a common commensal inhabitant of the guts of humans and warm-blooded animals, in addition to being one of the most significant pathogens. (Kaper et al., 2004). It is a commensal that has a mutually beneficial relationship with its hosts and only causes disease when Shiga toxin *E. coli* (STEC) strains produces toxins (Allocati et al., 2013). The most frequent cause of diarrhoea, particularly in countries that are developing, is *E. coli* (Nataro and Kaper, 1998; Gomes et al., 2016; Khalil et al., 2018). Since *E. coli* frequently lives in the guts of warm-blooded animals, it is frequently exposed to antibiotics, providing a strong selection pressure that causes it to develop resistance to drugs that its host consumes (Esonu et al., 2023).

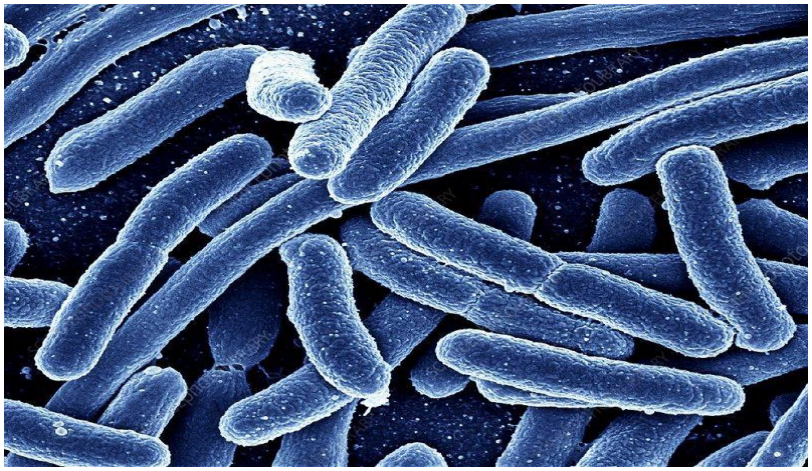


Figure 2.1: *E. coli* bacteria under a scanning electron microscope 7000X (https://media.sciencephoto.com/image/c0097438/800wm/C0097438Escherichia_coli_bacteria,_SEM.jpg).

Cultural Characteristics of *Escherichia Coli*

It grows at 37°C and is a facultative anaerobe (Pedraz et al., 2020). Depending on the type of growing medium utilised, isolates can have various appearances. For example, on nutrient agar, they can be big, thick, smooth, greyish white, opaque, or opaque discs (Koru and Ozyurt, 2008). On the contrary, lactose fermentation causes isolates on MacConkey agar medium to turn bright pink (**Figure 2.2**). While the rough form frequently auto-agglutinates in saline, the smooth form, which is visible in fresh isolation, is easily emulsified in saline. "Mucoid" colonies may occur in certain strains (Hiremath, 2018).

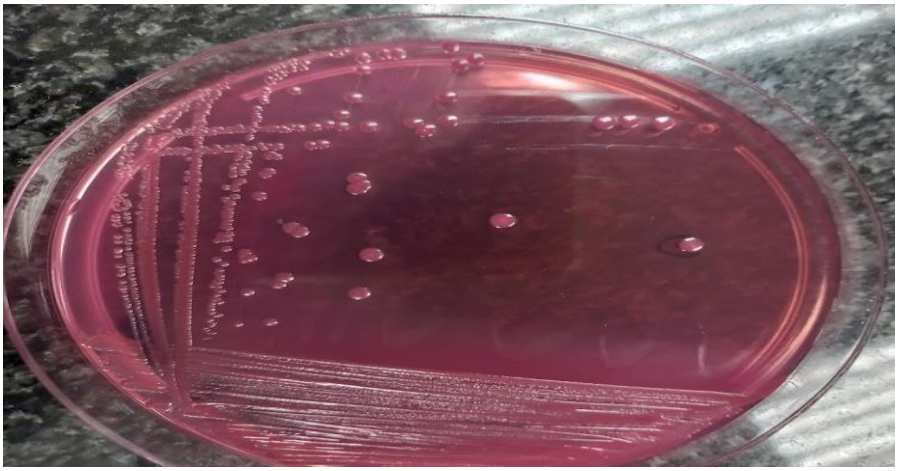


Figure 2.2: Growth of *E. coli* on MacConkey agar plate.

Mode of transmission of *E. coli*

The primary method of transmission between individuals is through physical touch (Jung et al., 2023). There is a significant danger of infection and transmission when food is handled or eaten with dirty hands (Alum et al., 2016). Transmission of *E. coli* can occur via close contact with infected animals or their surroundings (Persad and Lejeune, 2015). *E. coli* is released into the soil using manure and irrigation with sewage (Wang et al., 2020). Fatoba et al (2022) discovered high levels of *E. coli* in chicken litter-amended soil in uMshwathi Local Municipality, uMgungundlovu District, KwaZulu-Natal, South Africa, implying that the litter amendment resulted in soil contamination with *E. coli.*

2.3.1 OVERVIEW OF *E. coli* PATHOTYPES

Based on their virulence characteristics, enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC), and enterohaemorrhagic *E. coli* (EHEC) strains have been classified into six groups (Kaper et al., 2004). Additionally, *E. coli* is a significant source of resistance genes, which in both human and veterinary medicine can contribute to treatment failures (Poirel et al., 2018). In the enterobacterial gene pool, *E. coli* functions as both a donor and a recipient of antimicrobial resistance genes, allowing it to both receive and transfer resistance genes from other bacteria (Poirel et al., 2018).

Enterohemorrhagic *E. coli*/STEC

EHEC/STEC are a well-known group of foodborne microorganisms found all over the world (Kann et al., 2020). The fundamental virulence characteristic of the pathogroup is its capacity to manufacture one or more cytotoxins from the Shiga toxins (Stx) family (Makarova, 2023). EHEC/STEC can cause a variety of illnesses, from mild diarrhoea that is not detectable to more serious symptoms including haemorrhagic (HC) and the development of haemolytic uremic syndrome (HUS), a potentially fatal illness (Oriaku, 2021).

Enteropathogenic *E. coli*

It is harmful and known to cause both acute and persistent diarrhoeal illnesses in young children under the age of 5 years old (Gomes and Gonzalez-Pedrajo, 2010).

The capacity of EPEC to form A/E lesions is its most significant characteristic (Chen and Frankel, 2005). This allows bacteria to cause localised lesions by adhering firmly

to the intestinal epithelial cell's surface, disrupting the cell surfaces, and causing the microvilli to be destroyed (Chen and Frankel, 2005).

Enteroinvasive *E. coli*

In tests for lysine decarboxylase and lactose fermentative activity, enteroinvasive *E. coli* is non-motile negative. It penetrates human colon cells and produces an infection similar to that brought by *Shigella* species (Lali, 2018). Dysentery caused by EIEC is characterised by cramps, fever, and faeces that contain pus, mucus, and blood (Kaper et al., 2004). Once a bacteria is consumed, its adhesion proteins penetrate epithelial cells, resulting in diarrhoea and the presence of mucus and blood in stool specimens from the individuals affected (Mirsepasi-Lauridsen et al., 2019).

Enterotoxigenic *E. coli*

Enterotoxigenic *E. coli* (ETEC) is a varied strain of diarrhoeagenic bacteria that can target receptors in the small intestine by generating and releasing heat-labile (LT) and/or heat-stable (ST) enterotoxins (Fleckenstein, 2013). Several nations in eastern and central Africa, according to recent estimates, have a significant ETEC and *Shigella* burden, with children under the age of five from this regions having the highest ETEC mortality rates in the world (Khalil et al., 2018).

Enteroaggregative *E. coli* (EAEC)

Adhesins, toxins such as Shiga-like toxin, and other virulence proteins are encoded by highly variable EAEC genes. The *aggR* gene regulates the aggregative adhesion fimbriae I, which aid in intestinal colonisation. Additionally, according to Rivas et al. (2015), it produces entero-aggregative heat stable enterotoxin (EAST I toxin). The disease's manifestations include persistent and severe diarrhoea, which is especially prevalent in developing nations (Ugboko et al., 2020). EAEC is recognised as an arising intestinal illness that causes chronic diarrhoea in children residing in areas where it is prevalent, HIV-positive adults, and tourists from developed countries touring countries that are developing (Okeke and Nataro, 2001).

2.4 ANTIBIOTIC USE AND RESISTANCE IN CHILDREN

Antibiotics can save children's lives when they have bacterial illnesses, and they are the most prescribed therapy among all medications provided (Nicolini et al., 2014).

Antibiotics can cause adverse events such as drug toxicity and disrupted gut microbiota and weak immune system (Dethlefsen et al., 2008). Furthermore, antibiotic misuse promotes the emergence and dissemination of antimicrobial resistance at both the individual and population levels (Bebell et al., 2014). Selection and mutation can result in the emergence of resistance to antibiotics. For instance, some bacteria have evolved to produce enzymes that deactivate antibiotics, while others have developed biochemical pumps that can remove antibiotics before they reach their target (Allcock et al., 2017). The large incidence of bacterial causes of diarrhoeal disease in low-resource settings has led to demands for antibiotics to be used more widely for the treatment of diarrhoea even in the absence of dysentery (Rogawski et al., 2022).

Antimicrobial resistance is becoming increasingly problematic, prompting concerns that humanity could be heading back to a period where common infectious illnesses can no longer be effectively treated with treatments (Millar et al., 2001). Children have high antibiotic use rates and under-developed immune systems, making them a significant potential reservoir of antibiotic resistant bacteria in the community (Messina et al., 2020). Unsanitary living conditions and exposure to contaminated or inadequately treated water exacerbate the antimicrobial resistance issue in underdeveloped countries, particularly in rural areas (Hartinger et al., 2021). *E. coli* is also considered as an antibiotic resistance indicator bacterium (Varga et al., 2008). The environment, particularly water sources, and animal and human faecal flora act as natural habitats and sources of antibiotic-resistant bacteria (Larsson and Flach, 2022). Purohit et al (2017) discovered comparable and widespread antibiotic resistance, co-resistance, multi-drug resistance (MDR), and their genetic makeup in commensal bacteria from humans and their environments. Humans have a larger percentage of antibiotic resistance than the environment, although the load (number of resistant isolates/sample) is elevated in the environment (Purohit et al., 2017).

2.5 MECHANISMS OF RESISTANCE AND ARGs

Antibiotics are antimicrobial drugs that are utilised to treat and prevent bacterial illnesses caused by bacterial pathogens (Fymat, 2017). Antibiotic resistance poses a serious threat to food security, global health, and development. Critical, high, and medium priority are the three categories into which the WHO list is divided due to the urgent the need for new antibiotics (WHO, 2017).

Antibiotics are drugs that are used to kill bacteria (Etebu and Arikekpa, 2016). Bacteria, on the other hand, have a natural defence mechanism that permits them to resist antibiotics (Reygaert, 2018). Gene changes (mutations) activate the resistance mechanism (Abebe et al., 2016). Antibiotics create selection pressure, which leads genes to respond to the selection pressure (Wistrand-Yuen et al., 2018). Antibiotics with broad spectrum of action are frequently prescribed in hospitals to treat nosocomial infections; however, this increases resistance (Scaletsky et al., 2010). The usage of antibiotics is clearly promoting the emergence of resistance (Sengupta et al., 2013). On mobile genetic components, genes can be passed on from plasmids or obtained from plasmids (Partridge et al., 2018).

Horizontal gene transfer (HGT) allows antibiotic resistance to be transmitted down between bacterium species (Von Wintersdorff et al., 2016). Misuse of antibiotics or incorrect prescriptions also help facilitate the dissemination of resistance microbes (Saini et al., 2014).

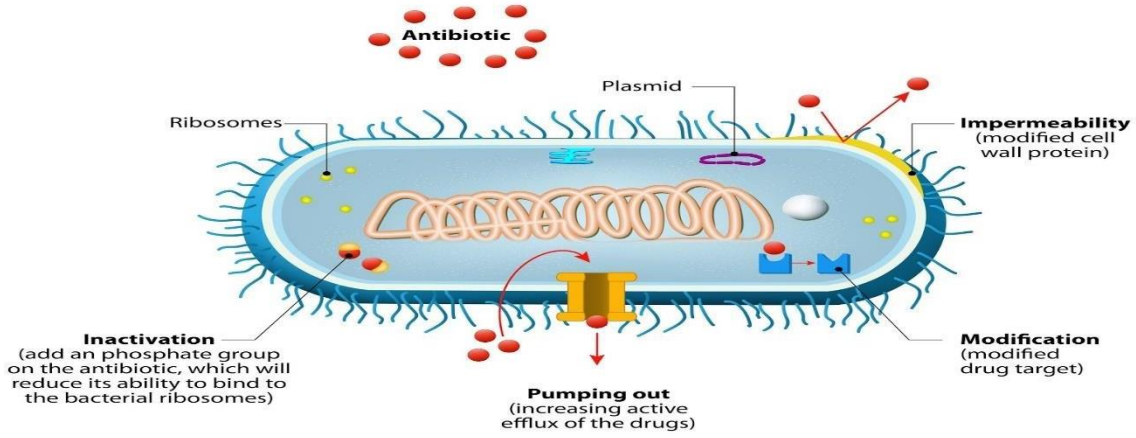


Figure 2.3: Antibiotic resistance mechanisms in bacteria (Zhivich, 2017).

B-LACTAM ANTIBIOTICS

Antibiotics known as β-lactams are bactericidal substances that provide an autolytic effect in addition to prevent the formation of the bacterial cell wall (Peris-Vicente et al., 2022). β-lactamases are present in some species of bacteria naturally; however, they have become widely distributed because of the overuse of β-lactam antibiotics and their distribution on plasmids (Castanheira et al., 2021). β-lactam antibiotics are utilised in the treatment and control of illnesses caused by bacteria (Pandey and

Cascella, 2019). β-lactam antibiotics function as covalent inhibitors against penicillin binding proteins (PBPs) by acylating a catalytically significant serine residue with the reactive β-lactam core (Mora-Ochomogo and Lohans, 2021). The widespread use of β-lactams antibiotics (such as ampicillin, amoxicillin, cephalexin) has led to the detection of Gram-negative bacteria that produce *TEM* and *SHV* in *E. coli* (originally in *K. pneumoniae*) (Gutkind et al., 2013).

2.5.1 MECHANISM OF β-LACTAM ANTIBIOTICS

The β-lactam ring plays a vital function in the mechanism of action of these drugs, which bind the enzymes involved in cell wall formation to target and block it (Zango et al., 2019). These enzymes are collectively known as PBPs and are anchored in the cell membrane (Goffin and Ghuysen, 1998). PBPs come in four to six different varieties depending on the species of bacteria. Transpeptidases, or PBPs involved in cell wall cross-linking, are frequently the most important for survival (Macheboeuf et al., 2006). The three-dimensional structure of β-lactam antibiotics is modelled by their 4-member ring, which functions as a natural substrate for transpeptidase activity during the formation of cell wall peptidoglycan (Avinash, 2015). Cell wall production is inhibited by the tight binding of these β-lactam antibiotics to the transpeptidase active site (Bhattacharjee, 2022). A cell may die from osmotic instability brought on by improper synthesis of its cell wall or by the β-lactam binding to PBP, which can set off a chain of events that leads in autolysis and cell death (Bhattacharjee, 2022).

2.5.2 CEPHALOSPORINS

Cephalosporins are β-lactam antibiotics that originated from the fungus *Acremonium,* formerly known as *Cephalosporium* (Shahbaz, 2017). Similar to other β-lactam antibiotics, cephalosporins are antibiotics that hinder the formation of the peptidoglycan layer that forms the bacterial cell wall (Mehta and Sharma, 2016). Reduction in the affinity of the current PBP components or acquisition of an additional PBP that is insensitive to β-lactam antibiotics are two ways that resistance to cephalosporin antibiotics can develop (Livermore, 1987). Their susceptibility to βlactamases is lower than that of other β-lactam antibiotics, such as penicillins. These antibiotics are currently resistant to several strains of *E. coli* (Petri, 2006).

2.5.3 MONOBACTAMS

Monobactams are β-lactams with a distinctive structure that includes a monocyclic nucleus (Balsalobre et al., 2019). Aztreonam, the only monobactam antibiotic available today, was first isolated from *Chromobacterium violaceum* and later modified (Tunkel, 1990). Aztreonam prevents peptidoglycan crosslinking, which kills bacteria and prevents the formation of cell walls (Baran et al., 2023).

2.5.4 CARBAPENEMS

β-lactam antibiotics include carbapenems as a subclass. These antibiotics are broad-spectrum. The following are examples of carbapenems: ertapenem, imipenem, doripenem, and meropenem (Sobia et al., 2022). The first line of treatment for illnesses caused on by highly resistant bacteria, such those caused by *E. coli*, is considered to be carbapenem antibiotics (Aurilio et al., 2022).

2.5.5 PENICILLINS

The second antibiotic that has been found is penicillin, which is the first to occur naturally (Aminov, 2010). A large family of bicyclic penam compounds, which vary in the type of acyl side chain linked to the fused β-lactam–thiazolidine ring system, make up the penicillin class of antibiotics (Bush, 2010).

2.5.6 EXTENDED BETA-LACTAMASE FAMILIES

Extended-spectrum β-lactamases (ESBLs) share biochemical properties, such as breakdown of β-lactam antibiotics and clavulanate inhibition (Paterson & Bonomo, 2005). However, the genes that encode these enzymes are heterogeneous and classified into distinct groups (Bradford, 2001).

TEM

TEM-type β-lactamases primarily target Gram-negative bacteria (Castanheira et al., 2021). These enzymes are particularly widespread in a wide range of bacterial species, including *Klebsiella pneumoniae, Enterobacter spp*., and *E. coli*, among the bacteria of the Enterobacteriaceae family. These organisms have a high prevalence of *TEM*-type beta-lactamases (Nagshetty et al., 2021).

SHV

SHV enzymes are found in Enterobacteriaceae (e.g., *E. coli, Klebsiella pneumoniae*) and caused infections in hospitals and other healthcare facilities (Ghenea et al., 2022). *SHV* β-lactamases are considered to have originated from a chromosomal penicillinase found in *Klebsiella pneumonia* (Livermore, 1995). They now include a significant variety of allelic variations. These variations include (ESBLs) (Livermore, 1995). These have a broad spectrum of hydrolysing action, including monobactams and carbapenems. Self-transmissible plasmids which often have resistance genes to other drug classes are encoded by *SHV-ESBLs* (Ahmed, 2021). They are now prevalent internationally in various Enterobacteriaceae, highlighting their clinical significance (Ghenea et al., 2022). These enzymes pose a difficulty as a result of their ability to easily spread between various kinds of bacteria (Ghenea et al., 2022).

CTX-M

The most prevalent ESBL group has been identified as *CTX-M* type enzymes, which have displaced *TEM* and *SHV* as the primary ESBL type (Castanheira et al., 2021). *E. coli* isolates usually contain enzymes of the *CTX-M* type (Girlich et al., 2020). Domestic animals, the environment, food products, livestock, and nosocomial and community settings have all been discovered to contain isolates with *CTX-M*-encoding genes (Liu et al., 2018). Treatment options are available for isolates that only contain these enzymes, but the efficacy of meropenem and other newer medications may be limited if these enzymes are combined with additional resistance mechanisms and create hydrolytic profiles with single amino acid mutations (Castanheira et al., 2021).

OXA

OXA-type β-lactamases are enzymes that contribute to antibiotic resistance (Evans and Amyes, 2014). Ambler class D and Bush-Jacoby-Medeiros functional group 2d enzymes are the categories for *OXA*-type β-lactamases, which hydrolyse oxacillin (Gutkind et al., 2013). *OXA*-type enzymes are mostly found in Enterobacteriaceae (e.g., *Escherichia coli*, *Klebsiella pneumoniae*) and *Pseudomonas aeruginosa*. These hydrolyse narrow-spectrum penicillin (Mwendwa, 2021). *OXA*-type β-lactamases are hard to identify in clinical laboratories due to their limited in vitro resistance to carbapenems (Akhtar et al., 2022).

β-LACTAM RESISTANCE

There are several ways in which resistance to the β-lactams might arise that have a link to the way these drugs work (Götte et al., 2017). β-lactams function as substitute substrates for penicillin-binding proteins (PBPs), which are essential for synthesising bacterial cell walls and facilitating the cross-linking of cell wall fragments prior to cell division (Kong et al., 2010). β-lactam antibiotic treatment affects the patient's microbiome, which could in the development of resistance in both the microorganism causing the infection and the normal flora's constituent organisms (Wiener et al., 1999). This impacts the individual's environment and other people. This implies that the development of resistance affects not only the hospital unit but also the healthcare providers and, consequently, their close associates (Wiener et al., 1999).

Antibiotics that target transmissible β-lactam-resistant organisms can have an impact on close groups of people, such as those in day care centres or institutions for long-term care (Kim et al., 2017). These microbes can be infectious agents or colonising bacteria that can simply move from the community to the hospital (Kim et al., 2017). Acquiring genes that express β-lactamases is the most common way that bacteria become resistant to β-lactam antibiotics (Jacoby, 1997).

2.6 TRANSMISSION PATHWAYS OF ANTIBIOTIC RESISTANT *E. coli* IN CHILDREN

Resistant bacteria circulate directly among humans as well as between humans, animals, and the environment within a community (Larson et al., 2019). Anthropogenic contamination causes the introduction of antibiotic resistance genes (ARGs) and antibiotic resistant bacteria (ARBs) into the natural environment (Stanton et al., 2022). The improper utilisation of antimicrobial drugs as productivity boosters in poultry or livestock is a major factor contributing to the spread of resistance to humans through animal products (Ahmad et al., 2021).

Elevated levels of microbiological markers of both faecal contamination and enteric infections have been found in studies conducted in developing countries on community member's hands (Parvez et al., 2019; Cantrell et al., 2023). According to reports, households without adequate access to sanitation are more likely to have human faeces on them (Pickering et al., 2012; Dickin and Gabrielsson, 2023. Additionally,

studies have shown that routine household tasks like sweeping, cooking, and dishwashing, as well as handwashing, may cause *E. coli* infection to increase rapidly (Bloomfield et al., 2012 ; Aunger et al., 2016).

Antibiotic-resistant microorganisms can be found in food, plants, animals, humans, and the environment (soil, water, and air) (Tamhankar et al., 2019). The excessive use and abuse of antibiotics, inadequate infection and disease prevention and control in healthcare facilities and agricultural settings, limited access to high-quality, reasonably priced medications, vaccines, and diagnostics, and a lack of knowledge and enforcement of the law are the primary causes of resistance to antibiotics (WHO, 2020).

2.7 A STRATEGY FOR FIGHTING ANTIBIOTIC RESISTANCE: SURVEILLANCE

Surveillance is the most essential methods of combating AMR (Gulumbe et al., 2022). The WHO released a worldwide strategy in May 2015 with the goal of making sure that antibiotics are of the best quality, work effectively, and are accessible to all individuals who need treatment (WHO, 2015). Individual countries are expected to develop their own national antimicrobial resistance plans in conformity with the WHO global strategy (WHO, 2015). Only a few member countries have antimicrobial laws in place (Iskandar et al., 2021). Antibiotic resistance surveillance and tracking of resistance trends by DNA sequencing are two actions that can aid monitor and reduce antimicrobial resistance globally (Hendriksen et al., 2019).

In several countries, including the United Kingdom, United States, South Africa, India, New Zealand, Vietnam and China, antibiotic resistance in diseases acquired in hospitals and communities is tracked and monitored (Avershina et al., 2021). Currently, laboratory-based monitoring is used in South Africa to gather information from the public and private sectors regarding the ESKAPE species (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter spp*.) (Ramsamy et al., 2018). The Group for Enteric, Respiratory, and Meningeal Disease Surveillance in South Africa (GERMS-SA), a national clinical microbiology network, provides data to the National Institute for Communicable Diseases (NICD) in addition to laboratory data obtained from public hospitals (Nyasulu, 2014).

The South African Society of Clinical Microbiology collects data from the private sector (Madeira, 2020). These data sets are gathered by the South African Antibiotic Stewardship Partnership (SAASP) and the South African Society of Clinical Microbiology (SASCM) (Madeira, 2020). In addition to increased rate of resistance in microorganisms that cause nosocomial and community-acquired illnesses, the WHO's 2014 report on antibiotic resistance surveillance also highlighted the lack of population-based AMR surveillance, which might have provided important data on the spread, deaths, and economic impact on community (WHO, 2014). It is quite challenging to comprehend completely how antibiotic resistance spreads because the several studies focus on tertiary hospitals, while studies that take community enrolment into account only enrol a small percentage of the population (Grundmann, 2014).

Effective surveillance requires the ability to identify and profile new antibiotic resistance routes (WHO, 2015). Antibiotic mechanism detection, on the other hand, demands specialized knowledge and technology; as a result, global antibiotic monitoring will not be produced overnight, but in stages (Grundmann, 2014). The world presently requires monitoring that creates high-quality data for thorough global planning and public health efforts to combat antibiotic resistance (WHO, 2014).

2.8 RISK FACTORS FOR RESISTANCE IN CHILDHOOD INFECTIOUS DISEASES

Abuse of antibiotics

Resistance is mostly driven by user-related reasons including self-medication, failure to comply, misinformation, and advertising pressures, as well as external factors like ignorance, lack of knowledge, and lacking access to health services and testing facilities (Byarugaba, 2004). Because individuals living in poverty lack access to hygienic practices and clean water, their chances of contracting infections are higher (Pal et al., 2018). Poor people are also at risk of treatment failure due to monetary problems and have inadequate basic nutritional status for fighting off infections (Byarugaba, 2004). Lack of diagnostic facilities, unprofessional conduct, and inadequate training are other aspects of healthcare practitioners that could result in improper prescription, which could lead to the wrong antibiotic being recommended (Byarugaba, 2004).

Quality of antibiotics

A lack of regulation for drugs causes developing countries to suffer from the availability of low-quality and counterfeit antibiotics (Kimura et al., 2020). When these inefficient antibiotics are used, resistance increases and more expensive, potent antimicrobials must be purchased with the intent of treating resistant strains (Roope et al., 2019).

2.9 UPDATE TO TECHNOLOGICAL ADVANCES TO AMR DETECTION AND MONITORING

Technological developments have greatly improved antimicrobial resistance (AMR) detection and monotoring allowing for more thorough, accurate and quick surveillance (Yamin et al, 2023). Antimicrobial susceptibility testing is the most widely used method because of its low cost and ease of performance and applicability of numerous bacterial species and antibiotics (Gajic et al., 2022). Molecular techniques have been used for the environmental monitoring of AMR genes in soil samples and has been shown to be a good way to find point mutations in broad-spectrum AMR genes, as long as either of the primers is designed to bind at sites where the sequence changes (Galhano et al., 2021).

2.9.1 ANTIBIOTIC SUSCEPTIBILTY TESTING

To confirm susceptibility towards particular antibiotics or detect resistance in individual bacterial isolates, antimicrobial susceptibility tests are important (Reller et al., 2009). The ability to grow these microorganisms in the lab using suitable growth media, or "culture," has made it possible to ascertain the susceptibility and resistance of specific infections to a variety of antimicrobial agents. This has allowed medical professionals to promptly start treating patients according to the recommended regimens (Lagier et al., 2015).

DISK DIFFUSION TEST

The Mueller-Hinton agar plate surface must be inoculated with 1 CFU/ML of bacteria using the simple, well-standardized disk diffusion susceptibility method (Butassi et al., 2017). On inoculated agar plates, many commercially fixed concentrations of paper antibiotic discs are placed. Prior to determining results, plates are incubated for 24 hours at 35 °C (Benkova et al., 2020). The millimetres of the growth inhibition zones are then identified. The diameter of the zone relates to both the drug's rate of diffusion through the agar media and the isolate's susceptibility (Hindler and Munro, 2010). The

guidelines released by the Clinical Laboratory Standards Institute (CLSI, previously the National Committee for Clinical Laboratory Standards or NCCLS) are used to evaluate the zone diameters of each drug (Jenkins & Jerris, 2011). Disk diffusion test results are "qualitative" because they are based on the test rather than a minimum inhibitory concentration (MIC), and can be classified as susceptible, intermediate, or resistant (Tenover, 2017). There is no specific equipment needed for the disk approach, which makes it easy to use (Reller et al., 2009).

BROTH DILUTION TEST

It's considered the earliest techniques for determining an antibiotic's susceptibility. It involves preparing two-fold dilutions in test tubes filled with a liquid growth media (Chitra, 2017). After overnight inoculation with a standardised bacterial suspension containing 1 CFU/mL, the antibiotic-containing tubes are examined for visible bacterial growth, which indicates a minimal inhibitory concentration (MIC) (Wiegand et al., 2008). The routine of manually preparing the antibiotic's serial dilutions accounts for a significant portion of the method's precision, which is plus or minus 1 two-fold concentration (Reller et al., 2009).

2.9.2 MOLECULAR IDENTIFICATION ASSAYS

Polymerase Chain Reaction (PCR)-based tests

The polymerase chain reaction (PCR) is an in-situ DNA replication method that takes into consideration the target DNA's rapid amplification when thermostable DNA polymerase and synthetic oligonucleotide primers are present (Point, 2014). Polymerase chain reaction is widely used for the quick detection, identification, isolation, and characterisation of microorganisms (Valones et al., 2009). PCR have been applied to the detection and diagnosis of infectious diseases, genetic fingerprint identification, DNA cloning, and infectious disease diagnosis (Rajalakshmi, 2017). PCR is useful in identifying common strains of bacteria that are present in coccoid forms that are viable but not culturable (Adzitey et al., 2013). Additionally, the application of PCR provides a safe distance from situations in which phenotypic characteristics are unclear and misinterpreted (Adzitey et al., 2012). Modern molecular biology technologies, such polymerase chain reaction (PCR) in conjunction with gel electrophoresis, which enhances detection effectiveness, are becoming increasingly important for the isolation and detection of bacteria (Umesha and Manukumar, 2018).

It can save time and money while simultaneously diagnosing several diseases because multiplex PCR has a higher detection efficiency than single PCR (Kadri, 2019).

Single Polymerase Chain Reaction

It is an identification method for organisms using polymerase chain reaction (PCR) with one primer pair (Joshi and Deshpande, 2010). Primers can be designed specifically for a specific species and are useful for identifying the target organism once it is surrounded by other organisms (Joshi and Deshpande, 2010). With or without pre-enrichment, this kind of PCR can be utilised to rapidly to directly identify or detect microorganisms in a specimen (Villamizar-Rodríguez et al., 2015).

Furthermore, direct selection of bacterium isolates from agar plates can be verified using a single PCR. Before sequencing, the 16S rRNA genes of bacteria are amplified using particular primers by PCR, which aids in the identification of novel or unknown bacterial species (Adzitey et al., 2012).

Multiplex polymerase chain reaction (m-PCR)

A number of primers are used in a single PCR mixture in the m-PCR, which is used to detect, identify, and distinguish microorganisms (Kalle et al., 2014). As a result, mPCR creates amplicons of various sizes that are unique for distinct DNA sequences by amplifying several target sequences in a single reaction (Kalle et al., 2014). Primer design is crucial in the creation of multiplex PCR, even though it lowers costs, restricts sample amount, and enables rapid identification of several bacterial species (Zhang et al., 2014). Multiple primers can interact with one another during the amplification process, all primers must have the same annealing temperatures, and the PCR products should be considerably distinct in sizes (Adzitey et al., 2013).

2.10 SUMMARY OF LITERATURE

Children in developing countries with inadequate sanitation have an average of four to eight diarrhoeal cases per year between the ages of one and two. Poor children in South Africa have a larger risk of dying from diarrhoea than their more developed peers (Kotloff et al., 2013; Ugboko et al., 2020; Poague et al., 2022). Soil serve as a source for microorganism found in faeces, contributing to contamination of faeces, drinking

water, hands, and food (Holcomb et al., 2020). Open defaecation, poor disposal of children faeces, poor wastewater management, and poor animal faecal management all contribute to soil pollution (Ngure et al., 2013; Giribabu et al., 2019). Water, sanitation, and hygiene are all linked to diarrhoea and malnutrition (Ngure et al., 2014; Shrestha et al., 2020). Children are a significant potential reservoir of antibiotic-resistant bacteria in the community due to their high rates of use of antibiotics and undeveloped immune systems (Pai et al., 2015).

In contrast to isolates that have not been exposed to antibiotics, the rate of resistant isolates of *E. coli* increases greatly when the bacteria are treated with ampicillin, norfloxacin, or kanamycin (Ranjbar and Sami, 2017). Antibiotic mechanisms are harder to determine, however effective surveillance of emerging antibiotic resistance routes is necessary (Uddin et al., 2021).

Bacteriocidal agents identified as β-lactam antibiotics are utilised to treat and manage illnesses by preventing the formation of bacterial cell walls (Pandey and Cascella, 2019; Peris-Vicente et al., 2022). Due to their widespread distribution on plasmids, β-lactam antibiotics have contributed in the identification of gram-negative bacteria that generate *TEM* and *SHV* genes in *E. coli* (Gutkind et al., 2019; Castanheira et al., 2021). Several elements such as ignorance, a lack of education, insufficient availability of medical services, self-medication, noncompliance, misinformation, and promotional pressures, aid in the emergence of antibiotic resistance (Chauhan et al., 2018). The risk of infection, poor nutritional status, and treatment discontinuation is greater in impoverished people. Inappropriate prescription by healthcare providers is a further concern (Chauhan et al., 2018).

The current investigation sought to identify *E. coli* antibiotic resistance patterns in children under five and their environment. The study concluded with the determination of the presence of *E. coli*, antibiotic phenotypic patterns, antibiotic resistance genes, and phylogenetic groups of *E. coli* in children's environments. The antibiotic susceptibility profiles of the samples was identified using the Kirby Bauer technique, whereas the antibiotic resistance genes and phylogenetic group of *E. coli* was determined using PCR.

Chapter 3
MATERIALS AND METHODS

3.1 STUDY SITE

The current study was carried out in the South African province of Limpopo province in Lwamondo village, which is outside of Thohoyandou (23.011°S 30.357°E) in the Vhembe District Municipality. There are 20 218 people that reside in this village (STATSSA, 2011). It is a rural area where farming is an essential activity. The people rear and keep domestic animals in their homes.

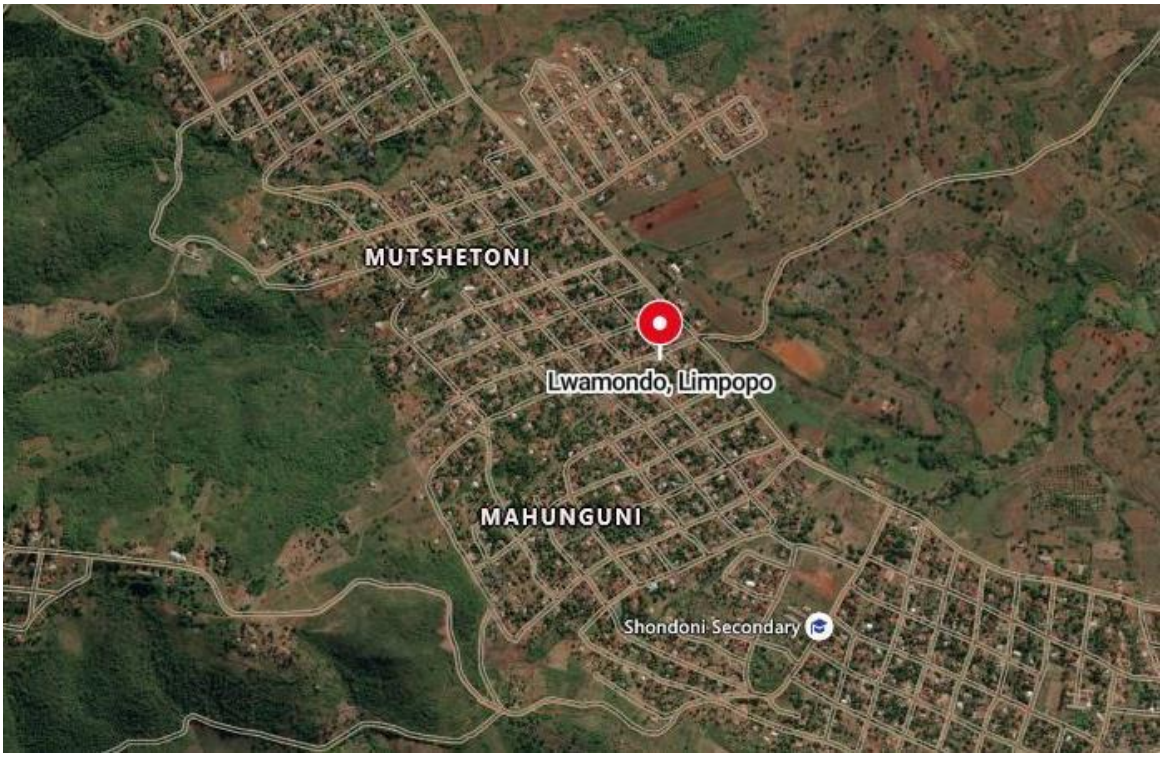


Figure 3.1: Satellite map showing the location of the sampling site Lwamondo village. (Map retrieved from https://www.google.co.za/maps).

3.2 ETHICAL CLEARANCE

Approval to conduct this study was authorised by the University of Venda's Research and Ethical Committee (SMNS/21/MBY/11/0504). The Lwamondo Village leaders gave permission to access the study location. Parents or guardians were assured of confidentiality and provided information about the study's goal and voluntary participation. All participants provided written informed consent, either personally or through their authorised competent guardians.

3.3 RESEARCH SUMMARY LAYOUT

The summary of the method used for this study is shown in **Figure 3.2** and the details of each method are described in the section below.

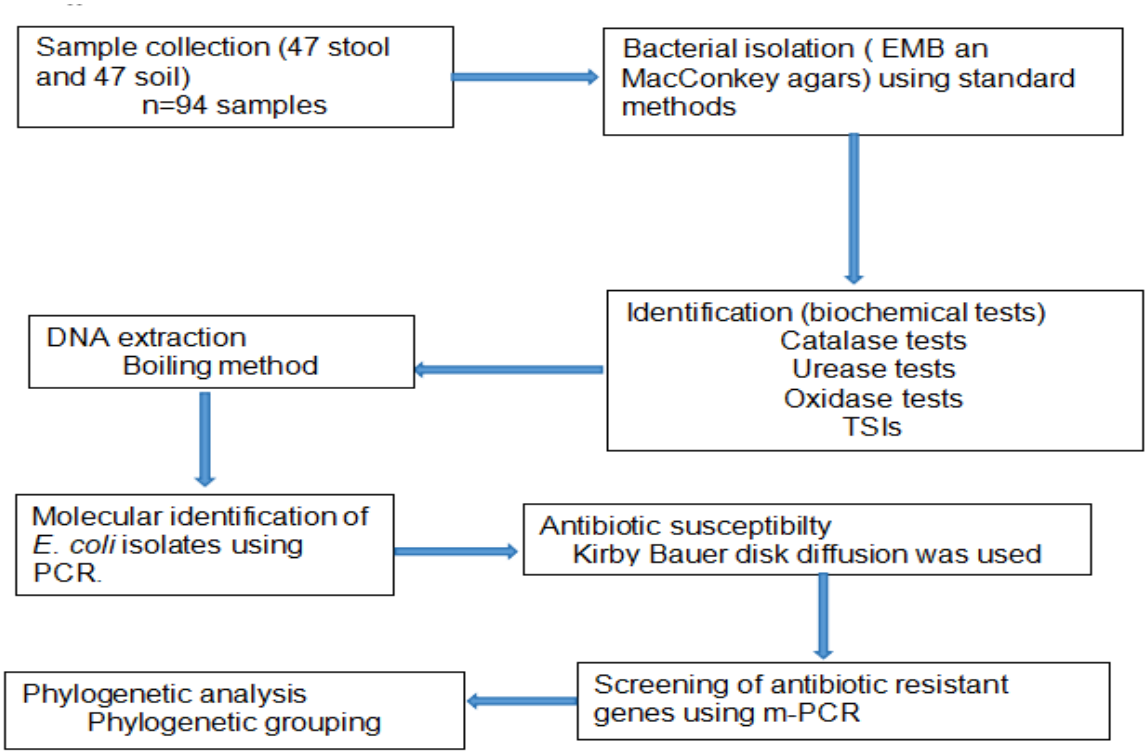


Figure 3.2: Flow diagram illustrating the methodical procedure used to detect *E. coli* in children's faeces and soil**.**

3.3 SAMPLE COLLECTION

The sample collection for this study was done from August 2022 to June 2023. Samples were collected from 18 villages and households were selected at random for sampling. Samples were collected by visiting households with children under 5 years asking for a fresh stool from morning diapers. A total of 47 stool samples were obtained using Compact Dry swabs (Carl Roth, Germany) from each of the children's diapers. From each household where the stool sample was collected, a soil sample was collected in the yard where the child usually plays. Each sample was labelled and stored in a cooler box with ice and transported to the University of Venda laboratory where laboratory analysis was performed. Upon arrival in the laboratory, samples were processed within 4 hours of collection.

3.4 PREPARATIONS AND ISOLATION OF SAMPLES FOR BACTERIAL ANALYSIS.

All media that were utilised in this study were prepared following manufacturer's instructions. In the laboratory, each stool specimen was aseptically enriched in peptone water buffer (Merck, Germany) overnight at 37°C. Thereafter, the enrichment of stool samples was cultured on Eosin methylene blue media (EMB) (HiMedia® Laboratories Pvt. Ltd, India) using the streaking plate technique. The plates were incubated for 24 hours. The next day, 3 isolates were randomly selected from each plate and sub-cultured on MacConkey (Merck, Germany) agar and incubated overnight at 37°C. To acquire pure isolates, the colonies from MacConkey agar (Merck, Germany) plates were sub-cultured on nutrient agar (Merck, Germany). Pure isolates were preserved in brain heart infusion broth (Mast Diagnostics, United Kingdom) in 20% (v/v) glycerol (Associated Chemical Enterprises (Pty) Ltd, Johannesburg, South Africa) and kept at -20°C until further analysis.

Soil samples were sieved using a fine mesh flour sifter sieve (Zhongbao Hardware Mesh Products Co, China) and a 1:50 dilution was prepared using phosphate buffer (Mast Group Ltd, United Kingdom). A volume of 0.5 ml was poured on EMB (HiMedia® Laboratories Pvt. Ltd, India) agar and spread using a sterile spreader and the plates were incubated for 24 hours at 37°C. Thereafter, three randomly selected isolates were and cultured on plates with MacConkey agar (Merck, Germany) and the plates were incubated. Nutrient agar (HiMedia® Laboratories Pvt. Ltd, India) was used to sub-culture isolates in order to obtain pure isolates. The selected pure isolates were preserved in heart infusion broth (Mast Diagnostics, United Kingdom) with 20% (v/v) glycerol (Associated Chemical Enterprises (Pty) Ltd, Johannesburg, South Africa) and stored at -20°C.

3.5 IDENTIFICATION OF *E. coli* ISOLATES

Four biochemical tests were conducted to further identify the isolates of *E. coli.* The isolates were identified utilising biochemical tests (Triple sugar iron agar, urease, oxidase, and catalase).

Triple sugar iron slant

In accordance with the protocol described by Lehman (2005), triple sugar iron (TSI) agar (HiMedia® Laboratories Pvt. Ltd, India) was utilised for further characterisation.

An isolate of *E. coli* is considered to be recognised by the presence of yellow slant, yellow butt, gas bubbles, and the lack of black precipitate in the butt (Sarba et al., 2019).

A new chosen colony was inoculated using a sterile inoculation needle. The process of inoculating the agar slant involves piercing through the middle of the medium to the tube's bottom, followed by streaking over the agar slant's surface. After sealing the test tube with cotton, it was permitted to incubate for an entire day.

Urease test

Using the technique described by Anyadoh-Nwadike et al (2017), this test was utilised to identify an organism's capacity to generate the urease enzyme, which break down urea. Two molecules of CO2 and ammonia are produced when urea is hydrolysed (Mekonnen et al., 2021). Phenol red's colour changes from a light orange at pH 6.8 to magenta at pH 8.4 indicates the presence of a pH shift. Ammonia reacts with CO2 and water to produce ammonium carbonate, which becomes moderately alkaline (Sapkota et al., 2023).

Urea agar slants (BioMeriux®, South Africa) were prepared in test tubes. A single pure colony from sub-cultured nutrient agar (HiMedia® Laboratories Pvt. Ltd, India) plates was inoculated inside a test tube using an inoculation loop and streaked on the slant minute. This was then placed on the incubator at 37ºC for 24 hours and the results were observed. Results were interpreted by observing the colour change after incubation where in, a shift in colour from strong magenta to a light pink in fifteen minutes to twenty four hours shows a positive result and no colour change after 24 hours showing a negative result (Mann, 1992).

Catalase test

According to Amoako et al (2015), this testing process indicates the existence of catalase, an enzyme assisting in the hydrogen peroxide's (H_2O_2) release of oxygen. It helps to distinguish bacteria that produce the catalase enzyme from those that lack it (Shaheen et al., 2021). A small, well-isolated colony was placed onto a clean glass slide using an inoculating loop. A drop of 3% H_2O_2 was placed onto the slide and thoroughly mixed. Bubbles were observed after 5 seconds. *E. coli* are catalase positive meaning bubbles forms when H_2O_2 is mixed with the colony.

Oxidase test

The oxidase method is utilised to determine the presence of tetramethyl-pphenylene-diamine, a redox dye, and cytochrome oxidase, an enzyme that catalyses and transfers electrons between electron donors in the bacteria, in the sample. The dye turns a rich purple colour at the end according to Ogodo et al (2022). Pure subcultured colonies of *E. coli* were used to conduct oxidase test. Oxidase test was done using the oxidase strips (Mast Diagnostics, United Kingdom). A sterile loop was used to place and rub the colony against the strip (Shields and Cathcart, 2013). Colonies that caused a colour change on the oxidase strip were recorded as oxidase positive and the opposite for colonies that did not cause colour change (Shields and Cathcart, 2013).

3.6 GENOMIC DNA EXTRACTION AND MOLECULAR IDENTIFICATION.

The boiling technique was utilised to extract all of the DNA from each isolate that tested positive for *E. coli*. One colony from a sixteen hour culture on Nutrient agar (HiMedia® Laboratories Pvt. Ltd, India) was inoculated in 250 μL of pure water. The suspension was heated at 100°C for 10 minutes and centrifuged at 13,000 rpm for 5 minutes, as described by Dashti et al (2009). For further analysis, the supernatant consisting the eluted DNA template was placed in a fresh, sterile, 2-milliliter centrifuge tubes and kept at -20°C.

Amplification of the *uidA* gene using PCR

The *uidA* gene was amplified by employing the extracted DNA as a template. The T100 heat cycler (Bio-Rad, USA) was used for molecular detection. **Table 3.1** contains a list of the primers utilised in the PCR test. 1 µM of each primer (1 L), 12 μL of Taq 2x master mix RED, 1.5 Mm MgCl2 (DNA-Gdansk, Poland), 3 μL of template DNA, and 8.5 μL of ultra-pure H_2O were all included in the PCR mixture, which had a total volume of 25 μL. The *uidA* gene's PCR conditions were chosen based on the primer's characteristics. The conditions used included five minutes at 94°C, thirty-five cycles (30 seconds at 95°C, 1 minute at 58°C, and 1 minute at 72°C), and 8 minutes at 72°C. The DNA of *E. coli* ATCC 25922 and the no template mix served as the positive and negative controls, respectively.

Table 3.1: Primer sequences for target genes and the size of PCR product of the genes.

Target genes	Primer Sequence (5'-3')	Amplicon size (bp)	References
uidA	F: AAA ACG GCA AGA AAAAGC AG R:ACGCGTGGTTAACAGTCTTGCG	147	Msolo et al., 2020
bla$_{TEM}$	F:AGTGCTGCCATAACCATGAGG R:CTGACTCCCCGTCGTGTAGATA	258	Naiemi et al., 2005
bla$_{CTX-M}$	F:GACAAAGAGAGTGCAACGGATG R:TCAGTGCGATCCAGACGAAA	319	Naiemi et al., 2005
bla$_{SHV}$	F:GATGAACGCTTTCCCATGATG R:CGCTGTTATCGCTCATGGTAA	381	Naiemi et al., 2005
bla$_{OXA}$	F:ATTATCTACAGCAGCGCCAGTG R:TGCATCCACGTCTTTGGTG	190	Sharma et al., 2013
chuA	F-GACGAACCAACGGTCAGGAT R-TGCCGCCAGTACCAAAGACA	279	Clermont et al., 2000
yjaA.1	F-TGAAGTGTCAGGAGACGCTG R-TGCCGCCAGTACCAAAGACA	211	Clermont et al., 2000
TspE4c2	F-GAGTAATGTCGGGGCATTCA R-CGCGCCAACAAAGTATTACG	152	Clermont et al., 2000

3.7 ANTIBIOTIC SUSCEPTIBILITY TESTING

E. coli isolates susceptibility to antibiotics was determined using the Clinical and Laboratory Standard Institute (CLSI) guidelines (CLSI, 2018). The Kirby Bauer disk diffusion method was utilised to assess each sample's pure and verified *E. coli* isolates antibiotic susceptibility. As shown in **Table 3.2**, each positive sample was tested against 14 antibiotics from various antibiotic classes that were chosen in accordance with the WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR). Nutrient agar (HiMedia® Laboratories Pvt. Ltd, India) was used to revive the confirmed isolates in glycerol stocks, and the samples were then incubated for twenty four hours at 37°C. *E. coli* isolates that were pure were combined with sterile water to create a suspension that matched the 0.5 McFarland standard (HiMedia® Laboratories Pvt. Ltd, India). The suspension was subsequently properly inoculated onto Mueller-Hinton agar (Oxoid, Basingstoke, UK) using sterile swab sticks. Antibiotic discs were then aseptically placed at equal distances using a disc dispenser. The plates were then incubated at 37°C for 24 hours and tested for sensitivity. Thereafter, a transparent ruler was used to measure each diameter of the clear zone surrounding the antibiotic in millimeters. The susceptibility testing uses empirical antibiotics used in the treatment of diarrhoea.

Table 3.2: List of antibiotics used for the current study.

Group	Antibiotic	Disc code	Disc Potency
β-lactam	Ampicillin	AP	10 µg
	Amoxycillin	A	25 µg
Cephalosporin	Cefotaxime	CTX	30 µg
	Cefoxitin	FOX	30 µg
Fluoroquinolone	Cephalexin	CL	30 µg
	Ciprofloxacin	CIP	5 µg
	Amikacin	AK	30 µg
Phenicol	Chloramphenicol	C	30 µg
Quinolones	Nalidixic acid	NA	30 µg
Carbapenem	Meropenem	MEM	10 µg
	Aztreonam	ATM	30 µg
Macrolide	Azithromycin	ATH	15 µg
Aminoglycoside	Gentamicin	GM	10 µg
Tetracyclines	Tetracycline	T	30 µg

3.8 ANTIBIOTIC RESISTANCE GENES SCREENING.

Using particular primers that target resistance genes (β-lactams), PCR was employed to analyse the isolate's antibiotic resistance genes according to observed phenotypic resistance profiles. As indicated in **Table 3.1**, m-PCR was carried out in a 25 µL reaction mixture that contained 1 µM (1 µL) of each forward and reverse specific primer combination.1, 8.5 µL of RNase-free water, 3 µL of DNA template, and 12 µL of 2X QIAGEN multiplex PCR master mix (3 Mm MgCl2, 3X 0.85 ml) (Qiagen, Hilden, Germany). The 1.5% agarose gel electrophoresis, which was visualised in a UV transilluminator (Gene Genius Bio Imaging system, Vacutec®) was used to verify the amplification of the PCR products. The PCR cycling conditions were as follows: 15 minutes at 95 °C; 30 cycles of denaturation at 95 °C for 40 seconds; 40 seconds of annealing at 56 °C; 50 seconds of extension at 72 °C; and a final 10 minutes of extension at 72 °C.

3.9 THE PHYLOGENETIC GROUPING OF *E. coli* ANTIBIOTIC RESISTANT GENES.

Utilising 2 genes (*chuA and yjaA*) and the DNA fragment *TSPE4.C2*, PCR was used to group *E. coli* isolates into several phylogenetic groups, as previously detailed by Cleremont et al (2000). It was employed to rapidly categorise isolates of *E. coli* phylogenetically in the following ways: 10 L of extracted DNA, 25 µL of Taq DNA Polymerase Master Mix RED (Ampliqon, Denmark), and 0.5 µL of each particular oligonucleotide primer (**Table 3.1)** were added to the reaction mixture, which had a total volume of 50 µL. The cycling conditions for the PCR were 10 minutes at 95°C, 35 cycles of denaturation at 95°C for 40 seconds, annealing at 57°C for 40 seconds, an extension at 72°C for 40 seconds, and a final extension lasting 8 minutes at 72°C. For each cycle, *E. coli* ATCC25922 served as the control strain.

3.10 ANALYSIS OF DATA

Spreadsheets developed with Microsoft Excel (365) were used to enter data from the PCR samples. Separate Excel spreadsheets were used to record questionnaire responses. All tables and graphs were created using Microsoft Excel (365) when necessary, and the total number of samples was calculated.

Chapter 4

RESULTS

4.1 DESCRIPTION OF THE SAMPLING SITE

The Lwamondo village in the Vhembe area, located outside of Thohoyandou, was selected as the study site. It has 22 sub-villages, and the villages have an estimated population of 20 000 inhabitants, mostly subsistence farmers. The study population concentrated on children under the age of 0 to 5 years and only 18 of sub-villages were considered. **Figure 4.1** depicts children's play area where a soil sample was collected. The play area is situated near where animals (pigs, goats, and chickens) were kept.

Figure 4.1: (Right) A children's play area surrounded by dry leaves, orange trees, and plastics, as well as an old pan in a household near an animal's house constructed of zinc, and (left) environmental conditions outside the house, having dirty-smelling laundry water flowing on topsoil, plastics, and zinc remainders.

4.1.1 DEMOGRAPHIC DATA OF STUDY POPULATION

In Lwamondo village, a total of 47 houses were enlisted to take part in the study from August 2022 and June 2023. The demographic information for the 47 children's households from which stool samples were taken is shown in **Table 4.1**. The study concentrated on households with children under 5 years old. About 40% (19/47) of the households had toddlers who were 2 years old. The least age groups participating in this study included children aged 0 years [4% (2/47)] and 5 years [2% (1/47)]. More males [65.96% (31/47)] were recruited in the study than female participants [34.04% (16/47)]. Out of 47 stool samples collected, 34.04% (16/47) of the children previously

reported having diarrhoea episodes, and 8 (17.02%) were treated with antibiotics. A total of 64.96% (31/47) of the children were not reported to have previously had diarrhoea. A total of 76.60% (36/47) of children were given antibiotics prior to collection of samples and 23.04% (11/47) were not given antibiotics. The questionnaires revealed that 5 (10.64%) of the parents had completed their higher education, 6.38% (3/47) had completed their primary education, 63.83% (30/47) had completed their secondary school, and 8.51% (4/47) did not have any form of schooling at all.

Table 4.1: The children's and their family member's demographic information.

Variables	Category	Total n=47 (%)
Date of birth of the child	2018	1 (2.13%)
	2019	6 (12.77%)
	2020	7(14.89%)
	2021	19 (40.43%)
	2022	12 (25.53%)
	2023	2 (4.26%)
Sex of the child	Female	16 (31.91%)
	Male	31 (65.95%)
Number of family members	Up to 5 members	22 (42.55%)
	6 or more members	25 (46.81%)
Maternal education	None	4 (8.51%)
	Primary	3 (6.38%)
	Secondary	30 (64%)
	Tertiary	5 (10.64%)
Illness (last 4 weeks)	Diarrhoea	16 (34.04%)
	No illness	31 (64.96%)
Given antibiotics	Yes	11 (23.04%)
	No	36 (76.60%)

4.1.2 SANITATION COVERAGE WITHIN HOUSEHOLDS

Table 4.2 illustrates the coverage of sanitation for children below five years. This study discovered that most of the household members used pit toilets 85% (40/47) and 15% (7/47) of the households used flush toilets. All participants [100% (47/47)] reported not sharing toilets with neighbours. About 68% (32/47) of parents reported that they cleaned their toilets 2-3 times a week; 15% (7/47) of the households said the toilets were cleaned every day; and 4% (2/47) of the toilets were never cleaned. Overall, 19% (9/47) of families that have children that share the same toilets as adults, and a total of 2% (1/47) families permitted children under the age of 5 years to engage in open defaecation, which gets disposed of in the pit toilets; however, this method does not

ensure the safe removal of all faeces from the surface. This could result in pathogenic bacteria contaminating the soil. The households that dispose of waste (food waste, papers, dried leaves) in the yard were 54% (24/47) and 2% (1/47) dispose of their waste far away from the house. The use of municipal waste collection was observed in 21% (10/47) of the households. About 34% (16/47) of participants dispose of their waste in a pit dug around the house. The conditions of children's alternative toilets were observed to be that during the sampling period, only 2% (1/47) of the children's alternative toilets were not clean, as indicated in

Figure 4.2, and only 2% (1/47) of the alternative children's toilets had an odour. This is a health issue because diseases can be transmitted directly (by contact) or indirectly (via airborne particles, vectors, and flies).

Table 4.2: Sanitation practices around households.

Variable	Category	Total n=47 (%)
Type of toilet	Pit Flush	40 (85%) 7 (15%)
Toilet sharing	Yes No	0 (0%) 47 (100%)
Cleaning of toilet	2-3 times a day Everyday Never	32 (68%) 7 (15%) 2 (4%)
Toilet sharing with children	Yes No	9 (19%) 38 (81%)
Alternative toilet for children	Open space in the yard Children's toilet Other	1 (2%) 1 (2%) 33 (70%)
Conditions of the alternative toilet for children	Clean Odour	1 (2%) 1 (2%)
Waste Handling	Yard Far away from the house Municipal waste collection Pit	24 (54%) 1 (2%) 10 (21%) 16 (34%)

Figure 4.2: The state of the children's alternative toilets.

4.1.3 HYGIENE PRACTICES

Practicing good hand hygiene is significant for preventing the transmission of pathogens and can contribute to reducing the frequency of diarrhoeal outbreaks (Taylor et al., 2015). Overall, the outcomes for the study population indicated that 100% (47/47) of the household members wash their hands, as indicated in **Table 4.3**. In Lwamondo village, most of the water source for hand hygiene practices came from tap water 96% (45/47). Only 4% (2/47) of the study participants used groundwater as a source, and no river water was used. A total of 53% (25/47) of the study population agreed that they use dishwashing containers as a hand-washing facility, and 21% (10/47) of the houses had a hand-washing facility next to the toilet. Households that use drinking beakers and water storage containers were 4% (2/47) and 6% (3/47), respectively.

The parents or guardians who reported practicing hand hygiene after using the toilet were 4% (2/47). Most of the parents answered that they wash their hands after changing nappies 45% (21/47), whereas parents who wash their hands before preparing meals and before meals were 11% (5/47) for both instances. As shown in **Table 4.3**, only 38% (18/47) of parents washed their hands after using the toilets, and only 23% (11/47) cleaned their hands after noticing that their hands were clearly dirty.

Table 4.3: Hygiene practices among parents/guardians of children under the age of five.

Variable	Category	Total n=47 (%)
Wash hands	Yes	47 (100%)
	No	0 (0%)
Water source for hand washing	Tap water	45 (96%)
	River water	0 (0%)
	Ground water	2 (4%)
Wash facility	Drinking beaker	2 (4%)
	Dishwashing container	25 (53%)
	Wash facility close to the toilet	10 (21%)
	Water storage container	3 (6%)
When do you wash	After using the toilet	18 (38%)
	After touching something contaminated	6 (13%)
	Before meals	5 (11%)
	Visibly soiled	11 (23%)
	Before preparing food	5 (11%)
	After changing nappies	21 (45%)

4.1.4 AGRICULTURAL PRACTICE IN HOUSEHOLDS

All the participants in this study practice agricultural activities such as animal keeping and cultivation of crops (**Table 4.4**). The household members showed 100% (47/47) in keeping animals and plant crops. A total of 57% (27/47) of the households grow plants and rear animals in their backyards, and about 43% (20/47) have farms not in the yard. Of the study population, 44% (21/47) of the participant applies organic fertilisers, 9% (4/47) apply inorganic fertilisers and 47% (22/47) do not apply any fertilisers before planting crops.

The type of organic fertilisers used was manure 44% (21/47). In most households, animals are allowed to roam around the yard 43% (20/47) and 57% (27/47) are allowed to roam outside the yard. In this study, the parents allowed children to play where animals were roaming (6%; 3/47) and a total of 17% (8/47) of the children were not allowed to play or touch the animals. In all households, 100% (47/47) of animal stools are disposed of in the yard and used as manure to help the soil to be fertile.

Table 4.4: Agricultural practices in selected households.

Variables	Category	Total n=47 (%)

Agricultural practice	Rear animals and plant crops	47 (100%)
Place of agricultural practice	Farms Yard	20 (43%) 27 (57%)
Disposals of animal stools	Around the yard Outside the yard	47 (100%) 0 (0%)
Use of fertilisers	Yes No	25 (53%) 22 (47%)
Type of fertilisers	Inorganic Organic (Manure)	4 (9%) 21 (44%)
Animal allowed to roam	Around the yard Outside the yard	20 (43%) 27 (57%)
Children allowed to with animals	Where animals are roaming Not allowed to play around the animals	3 (6%) 8 (17%)

Figure 4.3: Manure (animal's stools) applied on soil as an organic fertiliser ready for crop cultivation.

4.2 MORPHOLOGICAL CHARACTERISTICS AND APPEARANCE OF THE *E. coli* ISOLATES.

All collected stool and soil samples were cultured on MacConkey agar for the isolation of *E. coli*. Pink colonies on MacConkey agar were observed after 24 hours of incubation**.** Out of 216 isolates, 117 were isolates from stools, and 99 were isolates from the soil, which was presumptively identified as *E. coli*.

4.3 BIOCHEMICAL IDENTIFICATION OF THE BACTERIAL ISOLATES.

Bacteria isolated from the stool and soil sample were identified by different types of biochemical tests as indicated in **Appendix A**. Out of 216 isolates, 117 were from stools and 99 were from soil, which were biochemically verified to be *E. coli*.

4.4 CONFIRMATION OF *E. coli*

A single-plex PCR for assessing *E. coli* isolates was successful. **Figure 4.4** shows the PCR findings for specimens that tested positive for the *uidA gene*. Out of 216 presumptive *E. coli* isolates, the *uidA* gene was detected in 211 isolates (117 stool and 94 soil), confirming the presence of *E. coli.*

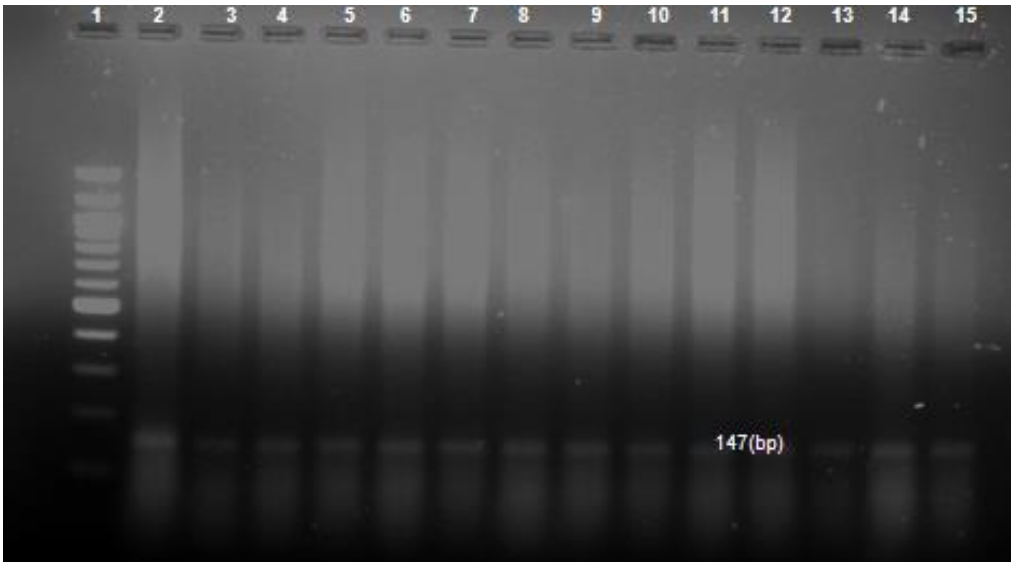
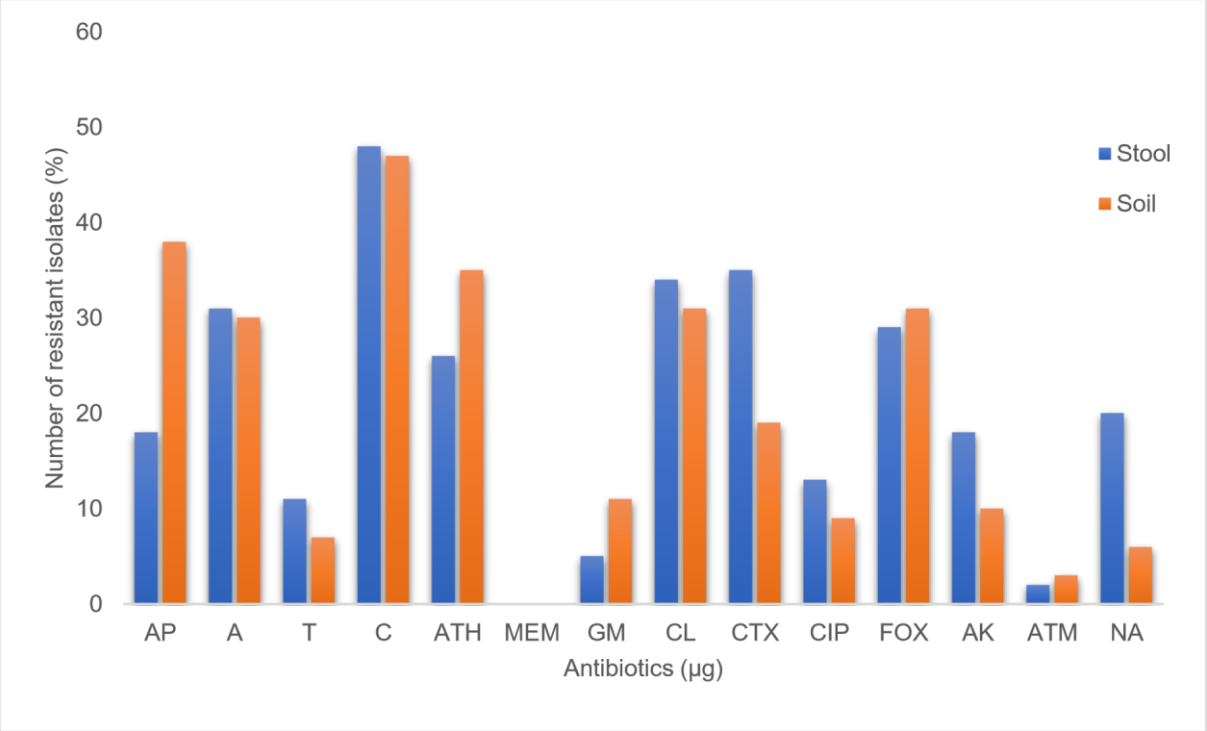


Figure 4.4: Gel electrophoresis image of the verified isolates of *E. coli* recovered from stool samples. Lane 1: DNA ladder (1200 bp), lane 2: negative control, lane 3: positive control (147 bp), lane 4: H1.1, Lane 5: H4.3, lane 6: H10.1, lane 7: H24.1, lane 8: H31.2, lane 9: 36.1, lane 10: S1.1, lane 11: S2.2, lane 12: S5.3, lane 13: S10.1, lane 14: S29.1 and lane15: S30.1.

4.5 ANTIBIOTIC RESISTANT *E. coli* AND MULTIDRUG RESISTANCE *E. coli.*

A total of 14 antibiotics were assessed on 117 stool isolates and 94 soil isolates. As seen in **Figure 4.5,** the highest detected phenotypic resistance of *E. coli* isolated from stools was 41% (48/117) chloramphenicol, cefotaxime 30% (35/117) and cephalexin 29% (34/117). The least detected phenotypic resistance was aztreonam 2% (2/117), gentamycin 4% (5/117), and tetracycline 9% (11/117).

As illustrated in **Figure 4.5** in soil, the greatest observed phenotypic resistance in *E. coli* isolates was chloramphenicol 50% (47/94), ampicillin 40% (38/94), and azithromycin 37% (35/94). Aztreonam 3% (3/94), nalidixic acid 6% (6/94), and ciprofloxacin 10% (9/94) showed the least phenotypic resistance. All isolates showed no resistance to meropenem. The overall results showed that there was an increase of phenotypic resistance in both faeces and soil samples to the antibiotic chloramphenicol.



Keywords: AP= Ampicillin, A= Amoxycillin, T= Tetracycline, C=Chloramphenicol, ATH= Azithromycin, MEM= Meropenem, GM= Gentamycin, CL= Cephalexin, CTX-M= Cefotaxime, CIP= Ciprofloxacin, FOX= Cefoxitin, AK= Amikacin, ATM= Aztreonam and NA= Nalidixic acid.

Figure 4.5: The comparison of *E. coli* resistant isolates against different β-lactamase antibiotics.

Appendix B demonstrates that 88 (75%) *E. coli* isolates tested positive for multidrug resistance. In this study, a total of 34.18% (40/117) isolates were resistant to two antibiotics detected in stool samples simultaneously. The highest observed multi-drug resistance with two antibiotics was ampicillin and cephalexin in 4 (3.42%) of the isolates. The most frequent combinations were seen in 2 (1.71%) of the isolates resistant to nalidixic acid, amikacin, and chloramphenicol.

In **Table 4.13**, soil samples, the multi-drug resistance was seen in 83 (88%) isolates. The most common combination of two antibiotics cephalexin + chloramphenicol was observed in 3.19% (3/94) isolates. The least detected multi-drug resistant combination was observed in 2.12% (2/94) of the isolates (cefoxitin + cephalexin + azithromycin and ciprofloxacin + cephalexin + azithromycin) and 1.06% (1/94) of the isolates (gentamycin + chloramphenicol and cefoxitin + tetracycline + azithromycin).

4.6 SCREENING OF ANTIBIOTIC RESISTANT GENES

Detection of β-lactamase resistant genes in *E. coli* was assessed for *bla*$_{TEM}$, *bla*$_{SHV}$ *and bla*$_{CTX-M}$, respectively (**Figure 4.6 and Figure 4.7)**. A total 211 (117 stool and 94 soil) confirmed *E. coli* isolates were for this were assessed for β-lactamase resistance. *bla*$_{TEM}$ was the most detected gene in 39% (46/117) of stool isolates. A total of 7% (8/117) isolates tested positive for the *bla*$_{SHV}$. Only 3% (4/117) *bla*$_{CTX-M}$ were detected in stools.

As depicted in **Figure 4.7**, the antibiotic resistant *E. coli* from soil were also positive for *bla*$_{TEM}$ and *bla*$_{SHV}$ genes. About 26% (24/94) of the isolates were positive for *bla*$_{TEM}$. Only 2% (2/94) of isolates had *bla*$_{SHV}$ gene in soil. The *bla*$_{OXA}$ gene in isolates was not detected in all samples. An overall total of 33% (70/94) *bla*$_{TEM}$ were positive in both soil and stool samples. *bla*$_{TEM}$ was the predominant antibiotic resistant gene in all samples selected. There were no *bla*$_{CTX-M}$ genes detected in soil.

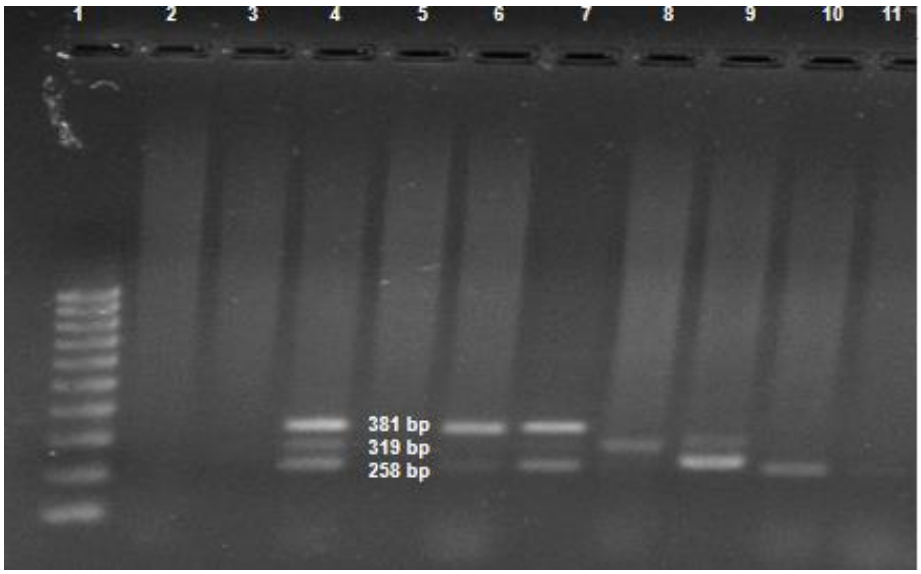


Figure 4.6: A gel electrophoresis of m- PCR products after amplification utilising specific primers. From left to right: lane 1: DNA ladder (1000 bp), lane 2: H1.1, lane 3: H3.1, lane 4:

H2.2 (*bla*$_{TEM}$ 258 bp, *bla*$_{SHV}$ 319 bp, *bla*$_{CTX-M}$ 381 bp), lane 5: S6.3, lane 6: H25.1 (*bla*$_{CTX-M}$ 381 bp & *bla*$_{TEM}$ 258 bp) , lane 7: H12.3 (*bla*$_{TEM}$ 258 bp ,*bla*$_{SHV}$ 319 bp & *bla*$_{CTX-M}$ 381 bp), lane 8: S10.1 (*bla*$_{SHV}$ 319 bp),lane 9: H9.3 (*bla*$_{TEM}$ 258 bp & *bla*$_{SHV}$ 319 bp), lane 10: S21.2 (*bla*$_{TEM}$ 258 bp) and lane 11: negative control.

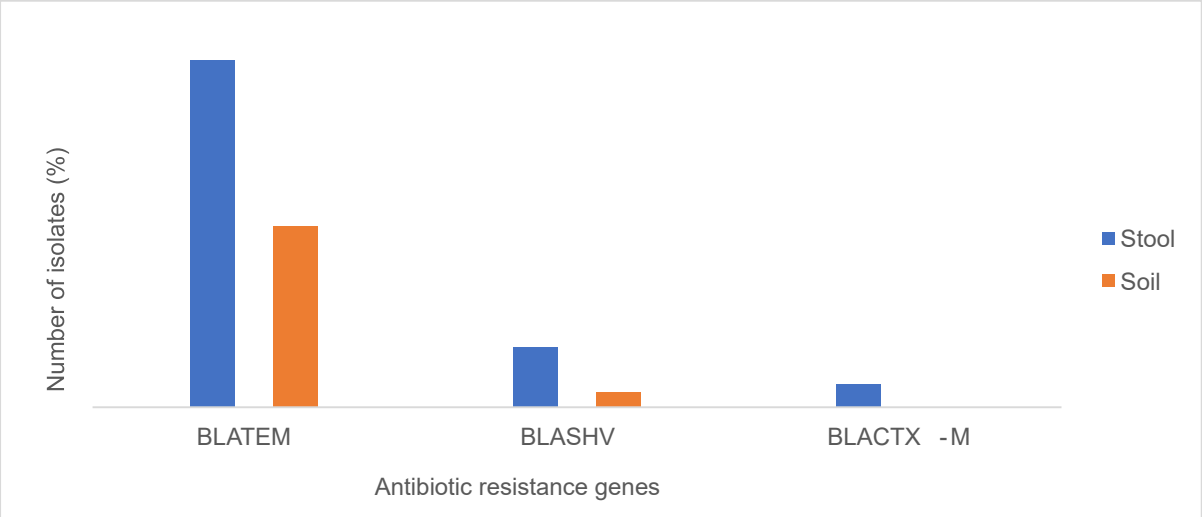


Figure 4.7: The distribution of *E. coli* antibiotic resistant genes in stool and soil.

As indicated in **Table 4.5**, the co-existence of antibiotic resistant genes patterns in soil were also detected in the study. In stool, only 2% (2/117) of the isolates were positive for 3 antibiotic resistant genes (*bla*$_{TEM}$ + *bla*$_{SHV}$ + *bla*$_{CTX-M}$). There were 3% (3/117) isolates were positive for both *bla*$_{SHV}$ + *bla*$_{TEM}$ and *bla*$_{TEM}$ + *bla*$_{CTX-M}$ was positive in 2% (2/117) of the isolates. In soil, the combination of two antibiotic resistance genes was detected. *bla*$_{SHV}$ + *bla*$_{TEM}$ was observed in 1% (1/94) of the isolates.

Table 4.5: The combinations of antibiotic resistant genes in stool and soil.

Antibiotic resistant gene	Stool n=117 (%)	Soil n=94 (%)
***bla*$_{TEM}$ + *bla*$_{SHV}$ + *bla*$_{CTX-M}$**	2 (2%)	0 (0%)
***bla*$_{SHV}$ + *bla*$_{TEM}$**	3 (3%)	1 (1%)
***bla*$_{TEM}$ + *bla*$_{CTX-M}$**	2 (2%)	0 (0%)
Total	**7 (7%)**	**1 (1%)**

The occurrence of antibiotic resistance genes in children by age group was assessed in this study **(Table 4.6)**. A total of 29% (2/7) of children under the age of 3 were positive for *bla*$_{SHV}$ + *bla*$_{TEM}$ and 8% (1/7) of the isolates were positive for *bla*$_{TEM}$ + *bla*$_{CTX-M}$

genes. At least 8% (1/12) child under the age of 1 was positive for *bla*$_{SHV}$ + *bla*$_{TEM}$ simultaneously. *bla*$_{TEM}$ + *bla*$_{CTX-M}$ was detected in a 5% (1/19) 2 years old child and *bla*$_{SHV}$ + *bla*$_{TEM}$ + *bla*$_{CTX-M}$ was also observed in a 11% (2/19) . There were no children under the age of zero with resistant genes.

Table 4.6: The distribution of antibiotic resistance genes in children according to age groups.

Antibiotic resistance genes	Age (number of isolates) n=47 (%)					
	0 year (n=2)	1 year (n=12)	2 years (n=19)	3 years (n=7)	4 years (n=6)	5 years (n=1)
Isolates with two ARG						
***bla*$_{SHV}$ + *bla*$_{TEM}$**	0 (0%)	1 (8%)	0 (0%)	2 (29%)	0 (0%)	0 (0%)
***bla*$_{TEM}$ + *bla*$_{CTX-M}$**	0 (0%)	0 (0%)	1 (5%)	1 (14%)	0 (0%)	0 (0%)
Isolates with three ARGs						
***bla*$_{TEM}$ + *bla*$_{SHV}$ + *bla*$_{CTX-M}$**	0 (0%)	0 (0%)	2 (11%)	0 (0%)	0 (0%)	0 (0%)
Total	0 (0%)	1 (8%)	3 (16%)	3 (43%)	0 (0%)	0 (0%)

Table 4.7 indicates the distribution of antibiotic resistant genes in different antibiotics in children's stool samples. Most isolates were resistant to chloramphenicol and positive for *bla*$_{TEM}$ gene 19.65% (23/117). The isolates resistant to azithromycin were positive for *bla*$_{TEM}$ 11.96% (14/117) and *bla*$_{CTX-M}$ 0.85% (1/117) genes. There were five isolates resistant to cefotaxime and positive for *bla*$_{SHV}$. At least 3.41% (4/117) isolates were resistant to cefoxitin and with *bla*$_{CTX-M}$ gene.

Table 4.7: Distribution of antibiotic resistant in different antibiotics in stools.

Antibiotics	Antibiotic resistant genes n=117 (%)		
	***bla*$_{TEM}$**	***bla*$_{SHV}$**	***bla*$_{CTX-M}$**
MEM	0 (0%)	0 (0%)	0 (0%)
FOX	10 (8.54%)	4 (3.41%)	4 (3.41%)
CTX	19 (16.23%)	5 (4.27%)	3 (2.56%)
AP	12 (10.23%)	4 (3.41%	2 (1.70%
GM	1 (0.85%)	0 (0%)	0 (0%)
C	23 (19.65%)	3 (2.56%)	0 (0%)
CIP	8 (6.88%)	2 (1.70%)	2 (1.70%)
AK	10 (8.54%)	1 (0.85%)	0 (0%)
T	6 (5.12%)	1 (0.85%)	0 (0%)
A	10 (8.54%)	3 (2.56%)	1 (0.85%)
NA	6 (5.12%)	1 (0.85%)	1 (0.85%
ATM	0 (0%)	0 (0%)	0 (0%)

CL	13 (11.11%)	3 (2.56%)	3 (2.56%)
ATH	14 (11.96%)	0 (0%)	1 (0.85%)
Total	**112 (95.72%)**	**27 (23.02%)**	**17 (14.48%)**

Keywords: AP= Ampicillin, A= Amoxycillin, T= Tetracycline, C=Chloramphenicol, ATH= Azithromycin, MEM= Meropenem, GM= Gentamycin, CL= Cephalexin, CTX-M= Cefotaxime, CIP= Ciprofloxacin, FOX= Cefoxitin, AK= Amikacin, ATM= Aztreonam and NA= Nalidixic acid.

In soil samples, isolates resistant to antibiotics and were positive for β-lactam genes were detected as shown on **Table 4.8**. There was a high number of isolates that were resistant to (19.15%; 18/94) chloramphenicol and *bla*$_{TEM}$ gene. Ampicillin, chloramphenicol, cephalexin, and azithromycin were found to be resistant to 1.06% (1/94) of isolate, and were positive for *bla*$_{SHV}$ gene, respectively. *bla*$_{SHV}$ and *bla*$_{CTX-M}$ were not identified in isolates resistant to gentamycin.

Table 4.8: Distribution of antibiotic resistance in different antibiotics in soil.

Antibiotics	**Antibiotic resistant genes n=94 (%)**		
	***bla*$_{TEM}$**	***bla*$_{SHV}$**	***bla*$_{CTX-M}$**
MEM	0 (0%)	0 (0%)	0 (0%)
FOX	5 (5.52%)	0 (0%)	0 (0%)
CTX	8 (8.51%)	0 (0%)	0 (0%)
AP	3 (3.19%)	1 (1.06%)	0 (0%)
GM	5 (5.32%)	0 (0%)	0 (0%)
C	18 (19.15%)	1 (1.06%)	0 (0%)
CIP	2 (2.13%)	0 (0%)	0 (0%)
AK	2 (2.13%)	0 (0%)	0 (0%)
T	3 (3.19%)	0 (0%)	0 (0%)
A	7 (7.45%)	0 (0%)	0 (0%)
NA	3 (3.19%)	0 (0%)	0 (0%)
ATM	3 (3.19%)	0 (0%)	0 (0%)
CL	4 (4.26%)	1 (1.06%)	0 (0%)
ATH	8 (8.51%)	1 (1.06%)	0 (0%)
Total	**71 (75.94%)**	**4 (4.24%)**	**0 (0%)**

Keywords: AP= Ampicillin, A= Amoxycillin, T= Tetracycline, C=Chloramphenicol, ATH= Azithromycin, MEM= Meropenem, GM= Gentamycin, CL= Cephalexin, CTX-M= Cefotaxime, CIP= Ciprofloxacin, FOX= Cefoxitin, AK= Amikacin, ATM= Aztreonam and NA= Nalidixic acid.

4.7 PHYLOGENETIC GROUPS DISTRIBUTION AMONG *E. coli* ISOLATES.

Figure 4.8 demonstrates the result of the PCR test for phylogenetic grouping of *E. coli* resistant isolates, which showed that the isolates were classified as A, B1, and D phylogroups. Only 75 isolates of *E. coli* harbouring antibiotic resistance genes were used in this test**. Table 4.9** indicates the distribution of phylogenetic groups of *E. coli*

in stool and soil samples. Isolates from stools 81% (38/47) belong to the group A. Group B1 was detected in 2% (1/47) isolate and 21% (10/47) belong to the group D. In soil, group A 96% (27/47) was detected as indicated in **Table 4.9.**

Table 4.9: The distribution of phylogenetic groups of *E. coli* in stool and soil.

Phylogroup	Stool n= 47 (%)	Soil n=28 (%)	Total n=75 (%)
A	38 (81%)	27 (96%)	65 (87%)
B1	1 (2%)	0 (0%)	1 (1.33%)
B2	0 (0%)	0 (0%)	0 (0%)
D	10 (21%)	0 (0%)	10 (13%)

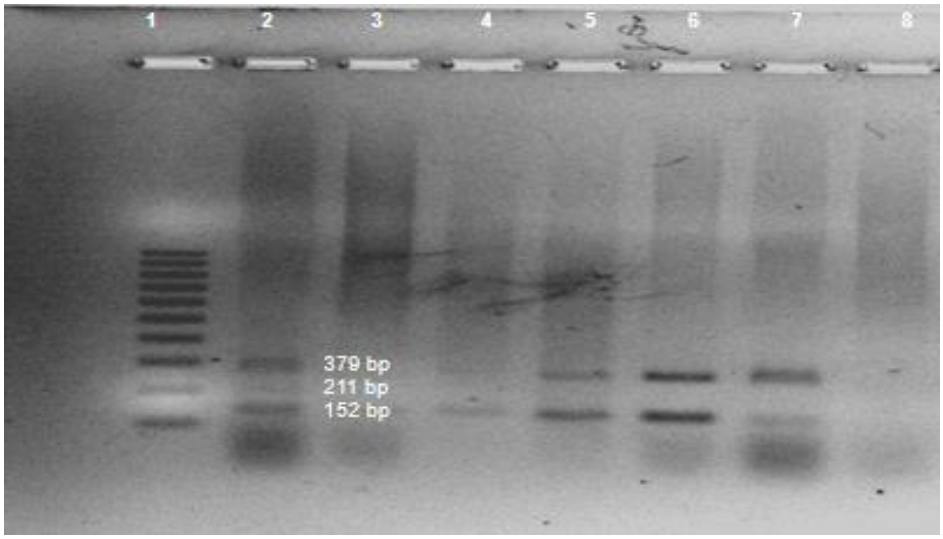


Figure 4.8: Gel electrophoresis of the multiplex PCR for the phylogenetic grouping of *E. coli.* Lane 1: DNA ladder (1000 bp); lane 2: positive control group B2 (*TspE4c2* 152 bp*, yjaA.1* 211 bp, *chuA 279* bp); lane 3: H25.1 [group B1 (*TspE4c2* 152 bp]; lane 4: H21.2 [group B1 (*TspE4c2* 152 bp)]; lane 5: H12.3[group D (*TspE4c2* 152 bp & *chuA* 279)]; lane 6: H16.3 [group D (*TspE4c2* 152 bp & *chuA* 279)]; lane 7: H2.3 [group D (*TspE4c2* 152 bp & *chuA* 279)] and lane 8: negative control.

Chapter 5

DISCUSSION AND CONCLUSION

5.1 DISCUSSION

Antimicrobial resistance (AMR) continues to pose a concern to world health (Maillard et al., 2020). In rural areas, children under 5 years old could be exposed to higher levels of infectious pathogens due to open defaecation, human faeces disposal, deteriorating latrines, or animal faeces in their environment when they play (Medgyesi et al., 2018). Young children are exposed to faeces when they ingest contaminated soil and objects from their yard and household (Ngure et al., 2013). The purpose of this study was to detect *E. coli* resistant to antibiotics in children and their environment.

The health status of children under five is greatly impacted by the educational background of their mothers (Desmennu et al., 2017). This current study reported a higher number of parents (64%) with secondary education. A previous study revealed that children of mothers who completed only primary school had a higher risk of diarrhoea than children of mothers with a higher level of education in Nigeria (Ugboko et al., 2021). This study had a higher percentage of children under the age of 1 (26%) 2 (40%), and 5 (2%) years. For growth and development, children under five rely on parents or guardians (WHO, 2004; Pem, 2015; Bornstein et al., 2022). Open defaecation was noted to still be practiced by 2% of children in this study because not all homes have access to sanitary facilities. Ledwaba et al (2019) reported that 30% of children were found to defaecate in open spaces in the yard in the Vhembe district, Limpopo. The availability of sanitary facilities minimises the probability of faecal-oral transmission and inappropriate disposal of excreta in homes and the community (Mills

et al., 2018). The diaper waste and other faecal materials should be disposed of properly to prevent the dispersal of faecal material into the household and environment (Majorin et al., 2014; Ellis et al., 2020). According to the WHO (2018), improper waste disposal increases the chance of pathogen contamination and dissemination. Improved sanitation can help reduce diarrhoeal illnesses and lower the morbidity rate of children under five, based on an investigation conducted in South Africa by Chola et al (2015). All parents/guardians reported to wash their hands in this study (100%). This is consistent with prior reports from carers of under-five children in rural countries such as Nigeria (Aigbiremolen et al., 2015).

Given that animal manure is regarded as a major source of both enteric and pathogenic antibiotic resistance genes and antibiotic resistant bacteria; using such waste from an animal that has not been treated to enrich soils for farming poses a risk of contamination (Johnson et al., 2016). Lwamondo village was selected as the location due to its high rate of antibiotic use, frequently without suitable medical supervision, as well as its considerable agricultural activities, which frequently involve the use of antibiotics in livestock and crops (Mthombeni et al., 2023). This study discovered that most households (44%) apply manure to cultivate their crops. Previous research examining the influence of manure from animals on soil resistance has demonstrated that the use of animal excrement in crop cultivation resulted in the increase in the quantity and variety of bacteria and genes resistant to antibiotics (Fatoba et al., 2021). Antibiotic resistance genes are significantly stored in animal manure because animal manure is frequently used in animal settings (Zhang et al., 2019).

A total of 47 samples were gathered respectively from soil and stool samples to provide the study with a total of 94 samples for assessment. Presumptive *E. coli* was isolated from 117 stool and 94 soil samples utilising biochemical and molecular methods. This is consistent with a study that reported that *E. coli* population numbers in soil and soil-related (manure) habitats have gradually declined in an open environment (Van Elsas et al., 2011). *E. coli* is less typically encountered in the soil (Solo-Gabriele et al., 2000) and water systems can induce diarrhoeal illnesses such as dehydrating diarrhoea and traveller's diarrhoea (Percival and Williams, 2014). In a different study done in rural North-West Ethiopia, 83% of soil samples collected from courtyards used for children's

play areas were positive for *E. coli* (Gizaw et al., 2022). The findings are consistent with those published by Chukwu et al (2019), who found that stool samples from both diarrhoeal and not having diarrhoea in children under five years old in the North-West province of South Africa had significant amounts of *E. coli*. Other studies have discovered that children in rural areas are subjected to intestinal pathogens through a variety of transmission routes, including contaminated water and food, contact with soil contaminated with animal faeces, and interpersonal contact (Wang et al., 2017; Medgyesi et al., 2018). The existence of *E. coli* in children's faeces and soil is a major concern for children who play on soil environments unsupervised (Medgyesi et al., 2018). In humans, *E. coli* has been identified as a major cause of bacterial illnesses, watery diarrhoea, and extraintestinal infections (Cabrera-Sosa and Ochoa, 2020).

The findings of this study reported that chloramphenicol (41%) was the most resistant antibiotic in soil samples than in stool samples (50%). Similarly, a previous study in rural Limpopo province, South Africa reported that *E. coli* isolates in children's stool with or without antibiotic exposure were less resistant to chloramphenicol (10%) (DeFrancesco et al., 2017). Omolajaiye et al (2020) revealed an elevated frequency of resistance against chloramphenicol in *E. coli* strains (94%) isolated from Amatole District Municipality of Eastern Cape, South Africa, which is corresponding to the findings of this study. In a study done by Msolo et al (2019), *E. coli* had a higher resistance rate to amoxicillin (95%) which is a health risk, and such amoxicillin-resisting isolates reported in this study were highly noted in both stool (31%) and soil (30%). Meropenem was not shown as a resistant antibiotic selected in the study. However, different studies reported that (98%) of *E. coli* isolates found in soil were resistant to meropenem in Kumasi, Ghana (Anokyewaa Appau and Ofori, 2024) and (96%) in Amathole and Chris Hani District Municipalities Eastern Cape Province, South Africa (Iwu et al., 2021). Similarly, a study done in Bangladesh discovered that household soil did not have any resistance to meropenem (Montealegre et al., 2018). The current study found (88%) multi-drug resistance to two or more antibiotics in stools, which is in agreement with previously reported finding in the Northwest province, South Africa by Chukwu et al (2019). In this study, 83% of the *E. coli* isolates were resistant to two or more antibiotics which is higher as compared to MDR *E. coli* from soil (10%) reported in Tanzania (Sonola et al., 2021). In KwaZulu-Natal, South Africa, Fatoba et al (2022) found a high rate of MDR *E. coli* in litter-amended soil (65%).

The occurrence of two or more antibiotics in stool was found in this study, ampicillin + cephalexin (3%), nalidixic acid + amikacin + chloramphenicol (2%) was the most common in the present study which differs with results reported by Bartoloni et al (2006) wherein ampicillin + tetracycline (78%) multi-drug resistant patterns were common in children's faeces from Bolivia, Peru. This highlights an alarming scenario in which routinely used antibiotics may not successfully cure infections caused by *E. coli*. The Vhembe district's multi-drug resistance to antibiotics could be caused by bacterial gene alterations that result in multi-drug resistant pathotypes, changes in disease control methods, or misuse of medicatio ns (Samie et al., 2007).

The bla_{TEM} gene was the predominant antibiotic resistant gene in stools of children aged from 1 to 5 years and soil with overall total of (33%). In Egypt, an identical investigation was done by Khairy et al (2020), who reported that bla_{TEM} gene was detected in 36% from children's stool samples. The genes bla_{TEM}, bla_{SHV}, and bla_{CTX-M} were not identified in the soil. Therefore, results are different from those reported by Graham et al (2016), who discovered the occurrence of bla_{TEM}, bla_{SHV}, bla_{OXA}, and bla_{CTX-M} genes in soil from Denmark and proposed that the existence of resistance genes in faeces from animals and people are linked. Only 1% of bla_{SHV} and bla_{TEM} genes in soil in this study were detected. This could be due to the frequency of antibiotic resistance genes (ARGs) could be greatly impacted by the pH, heavy metals in soil, mobile genetic elements, and the composition of microbial communities (Han et al., 2022). This study found that 7% and 1% of antibiotic-resistant *E. coli* isolates in faeces and soil contained two or more β-lactamase genes, respectively. The results were consistent with those reported by Ahmed and Asghar (2021) in Makkah, Saudi Arabia, where bla_{TEM} + bla_{SHV} + bla_{CTX-M} genes co-existed. In Port Elizabeth, South Africa, a study discovered the co-occurance of bla_{OXA-10} and bla_{TEM} genes in *E. coli* isolated from urine specimens (Gqunta and Govender, 2015). The bla_{TEM} + bla_{SHV} genes were the most frequent combination in the current study, which differs from a prior study that found these three genes (bla_{TEM} + bla_{SHV} + bla_{CTX-M}) in children from Khartoum, Sudan (Dirar et al., 2020). The study also found that the bla_{SHV} + bla_{TEM}, the bla_{TEM} + bla_{CTX-M}, and the bla_{TEM} + bla_{SHV} + bla_{CTX-M} genes were detected in samples of children under the ages of 1 (8%), 2 (16%), and 3 (43%) years. The study results corroborate with studies done that show that the gut microbiome of infants has a significant amount of antibiotic resistance genes (ARGs) from Northern California

(USA) (Casaburi et al., 2019; Samarra et al., 2023). In this study, beta-lactamase genes were not detected in children aged 0 (newborns). This is contradicting with a study that reveals that certain ARGs may even manifest in infants shortly after birth, most likely due to transmission from the mother or the hospital setting (Samarra et al., 2023). In this study *bla*$_{TEM}$ was the most frequent gene in isolates resistant to chloramphenicol in both stool (20%) and soil (19%). A study was conducted to show that *E. coli* isolated from Egyptian broiler farms was resistant to cephalosporins and penicillins primarily because it produced *bla*$_{TEM}$ β-lactamases, which are encoded by *bla*$_{TEM}$ genes (Abdel-Rahman et al., 2023).

As presented on **Table 4.9**, the predominant phylogroup discovered in the study was Group A (70%). The results correspond with those of Khairy et al (2020) in Upper Egypt, who observed that phylogroup A (47%) predominated among the isolates included in the study. The frequency of *E. coli* isolates from group B1 in the present study was 1%. In isolates from soil in Bangladesh (Bhowmik et al., 2023) and Brazil (Furlan and Stehling, 2021), studies found a significant presence of group B1. Human diarrhoeal illnesses have been linked to phylogroups A and B1 in Iran (Bailey et al., 2010). However, phylogroup B2 was not detected in this study. In Mexico Ahumada-Santos et al (2020) reported that phylogroup B2 *E. coli* is the most frequent in children's stools. Additionally, a study by Alfinete et al (2022) in Vhembe district, Limpopo, showed that phylogroup B2 was predominant in 30% of *E. coli* isolated from children's faeces. Since environmental strains appear to be more sensitive to climate and location, this apparent difference may indicate that these factors influence phylogroup distribution (Stoppe et al., 2017). According to several research, group A is admirably adapted to the environment (Skurnik et al., 2008; Anastasi et al., 2012).

5.2 CONCLUSION

The primary objective of this current study was to identify antibiotic-resistant *E. coli* in children residing in the rural community of Lwamondo village and the environment around them. This was accomplished by isolating *E. coli* from children's stool specimens and the surrounding soil. *E. coli* was found to be more predominant in children's stools than in soil samples. The confirmation of *E. coli* using the *uidA* gene was successful in both stool and soil samples. In this study, isolates of *E. coli* were resistant to 13 antibiotics but not meropenem. *E. coli* isolates from children were

shown to carry more *bla*$_{TEM}$ genes than other β-lactamase genes. The phylogenetic grouping of *E. coli* was achieved.

The study showed that children's stools and soil in Lwamondo village are highly contaminated with *E. coli.* The presence of these microorganisms indicates the soil in this setting is not safe for children to play unattended and there is a need to keep practicing proper hygiene. Parents and guardians need to follow instructions when giving their children antibiotics. Misuse of antibiotics and soil contamination and the practice of proper hygiene should be given more attention.

For a long time, rural communities have taken care of their environment, health, and livestock. This has changed as the population has grown and communities lack infrastructure. More frequent monitoring of the use of antibiotics in children is critical. Community members should stop the disposal of waste on soil and follow health professional's instructions when giving their children antibiotics. Government needs to collaborate with research institutions, pharmaceutical companies, and international organizations to develop new antibiotics specifically targeting diarrhoeal pathogens. Training the new moms and elderly people taking care of children on how to use antibiotics is needed. This is the first study in Lwamondo village to report on antibiotic resistance in *E. coli.* Overall, this study gave a new insight in the burden of antibiotic resistance *E. coli* carriage in children and their environment in Lwamondo village.

5.3 LIMITATIONS OF STUDY

The diarrhoeagenic strains were not characterised to evaluate their ability to cause diarrhoea. The did not comprehensively screen for all antimicrobial resistance genes. When community members learnt that stool and soil samples were required for the study, they found it difficult to participate. Some had concerns about potential cultural challenges, such as witchcraft rituals. The quantity of samples obtained was limited by the parent's absence during sample collection.

5.4 RECOMMENDATIONS

This study provides a better knowledge of antibiotic-resistant *E. coli* in children and the environment, as well as promoting awareness. The outcomes of this study will facilitate a deeper comprehension of the environment in Lwamondo Village and other comparable countries, as well as the spread of *E. coli* resistant to antibiotics in children.

Additionally, it will result in the creation of new monitoring systems to track and assess the spread of *E. coli* that is resistant to antibiotics and to reduce the widespread rise of this phenomena in the future. This highlights the significance of on-going surveillance for antibiotic-resistant genes and the phylogenetic distribution of *E. coli* isolates within this community and the broader South African region. To identify new sequence types and genes linked to antibiotic resistance, more research on the genotyping of pathogenic *E. coli* needs to be done.

The study provides a valuable reference for future research on the frequency of β-lactamase-producing *E. coli* in children and the environment. Future research should include all hospitals in the country to evaluate ESBL prevalence. Furthermore, understanding the antimicrobial resistance patterns and resistance genes of ESBL-producing *E. coli* infections aids in antibiotic resistance surveillance and control. Further research into multi-drug resistant *E. coli,* particularly ESBL, in children is critical in South Africa. This study adds to the evidence of bla_{CTX-M} and bla_{TEM}*'s* global spread and emphasises the significance of epidemiological surveillance. It is crucial to understand β-lactamases and their effects to control antibiotic use and prevent antibiotic resistance. To keep β-lactam antibiotics effective in treating serious infections, it is imperative to use them appropriately.

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APPENDICES

APPENDIX A: BIOCHEMICAL TESTING RESULTS

Biochemical tests (catalase, urease, oxidase, and triple sugar iron) were used to identify bacteria in children's stool samples.

Sample ID	Catalase	Urease	Oxidase	Triple sugar iron agar	Presumptive bacteria
H1.1	Positive	Negative	Negative	Positive	*E. coli*
H 1.2	Positive	Negative	Negative	Positive	*E. coli*
H1.3	Positive	Negative	Negative	Positive	*E. coli*
H2.1	Positive	Negative	Negative	Positive	*E. coli*
H2.2	Positive	Negative	Negative	Positive	*E. coli*
H2.3	Positive	Negative	Negative	Positive	*E. coli*
H3.1	Positive	Negative	Negative	Positive	*E. coli*
H3.2	Positive	Negative	Negative	Positive	*E. coli*
H3.3	Positive	Negative	Negative	Positive	*E. coli*
H4.1	Positive	Negative	Negative	Positive	*E. coli*
H4.2	Positive	Negative	Negative	Positive	*E. coli*
H4.3	Positive	Negative	Negative	Positive	*E. coli*
H5.1	Positive	Negative	Negative	Positive	*E. coli*
H5.2	Positive	Negative	Negative	Positive	*E. coli*
H5.3	Positive	Negative	Negative	Positive	*E. coli*
H6.1	Positive	Negative	Negative	Positive	*E. coli*
H6.2	Positive	Negative	Negative	Positive	*E. coli*
H6.3	Positive	Negative	Negative	Positive	*E. coli*
H7.1	Positive	Negative	Negative	Positive	*E. coli*
H7.2	Positive	Negative	Negative	Positive	*E. coli*
H7.3	Positive	Negative	Negative	Positive	*E. coli*
H8.1	Positive	Negative	Negative	Positive	*E. coli*
H8.2	Positive	Negative	Negative	Positive	*E. coli*
H8.3	Positive	Negative	Negative	Positive	*E. coli*
H9.1	Positive	Negative	Negative	Positive	*E. coli*
H9.2	Positive	Negative	Negative	Positive	*E. coli*
H9.3	Positive	Negative	Negative	Positive	*E. coli*
H10.1	Positive	Negative	Negative	Positive	*E. coli*
H10.2	Positive	Negative	Negative	Positive	*E. coli*
H10.3	Positive	Negative	Negative	Positive	*E. coli*
H11.1	Positive	Negative	Negative	Positive	*E. coli*
H11.2	Positive	Negative	Negative	Positive	*E. coli*
H11.3	Positive	Negative	Negative	Positive	*E. coli*
H12.1	Positive	Negative	Negative	Positive	*E. coli*

H12.2	Positive	Negative	Negative	Positive	*E. coli*

H12.3	Positive	Negative	Negative	Positive	*E. coli*
H13.1	Positive	Negative	Negative	Positive	*E. coli*
H13.2	Positive	Negative	Negative	Positive	*E. coli*
H13.3	Positive	Negative	Negative	Positive	*E. coli*
H14.1	Positive	Negative	Negative	Positive	*E. coli*
H14.2	Positive	Negative	Negative	Positive	*E. coli*
H14.3	Positive	Negative	Negative	Positive	*E. coli*
H15.1	Positive	Negative	Negative	Positive	*E. coli*
H15.2	Positive	Negative	Negative	Positive	*E. coli*
H15.3	Positive	Negative	Negative	Positive	*E. coli*
H16.1	Positive	Negative	Negative	Positive	*E. coli*
H16.2	Positive	Negative	Negative	Positive	*E. coli*
H16.3	Positive	Negative	Negative	Positive	*E. coli*
H17.1	Positive	Negative	Negative	Positive	*E. coli*
H17.2	Positive	Negative	Negative	Positive	*E. coli*
H17.3	Positive	Negative	Negative	Positive	*E. coli*
H18.1	Positive	Negative	Negative	Positive	*E. coli*
H18.2	Positive	Negative	Negative	Positive	*E. coli*
H18.3	Positive	Negative	Negative	Positive	*E. coli*
H19.1	Positive	Negative	Negative	Positive	*E. coli*
H19.2	Positive	Negative	Negative	Positive	*E. coli*
H19.3	Positive	Negative	Negative	Positive	*E. coli*
H20.1	Positive	Negative	Negative	Positive	*E. coli*
H20.2	Positive	Negative	Negative	Positive	*E. coli*
H20.3	Positive	Negative	Negative	Positive	*E. coli*
H21.1	Positive	Negative	Negative	Positive	*E. coli*
H21.2	Positive	Negative	Negative	Positive	*E. coli*
H21.3	Positive	Negative	Negative	Positive	*E. coli*
H22.1	Positive	Negative	Negative	Positive	*E. coli*
H22.2	Positive	Negative	Negative	Positive	*E. coli*
H22.3	Positive	Negative	Negative	Positive	*E. coli*
H23.1	Positive	Negative	Negative	Positive	*E. coli*
H23.2	Positive	Negative	Negative	Positive	*E. coli*
H23.3	Positive	Negative	Negative	Positive	*E. coli*
H24.1	Positive	Negative	Negative	Positive	*E. coli*
H24.2	Positive	Negative	Negative	Positive	*E. coli*
H24.3	Positive	Negative	Negative	Positive	*E. coli*
H25.1	Positive	Negative	Negative	Positive	*E. coli*
H25.2	Positive	Negative	Negative	Positive	*E coli*
H25.3	Positive	Negative	Negative	Positive	*E. coli*

H26.1	Positive	Negative	Negative	Positive	*E. coli*
H26.2	Positive	Negative	Negative	Positive	*E. coli*
H26.3	Positive	Negative	Negative	Positive	*E. coli*
H27.1	Positive	Negative	Negative	Positive	*E. coli*
H27.2	Positive	Negative	Negative	Positive	*E. coli*
H27.3	Positive	Negative	Negative	Positive	*E. coli*
H28.1	Positive	Negative	Negative	Positive	*E. coli*
H28.2	Positive	Negative	Negative	Positive	*E. coli*
H28.3	Positive	Negative	Negative	Positive	*E. coli*
H29.1	Positive	Negative	Negative	Positive	*E. coli*
H29.2	Positive	Negative	Negative	Positive	*E. coli*
H29.3	Positive	Negative	Negative	Positive	*E. coli*
H30.1	Positive	Negative	Negative	Positive	*E. coli*
H30.2	Positive	Negative	Negative	Positive	*E. coli*
H30.3	Positive	Negative	Negative	Positive	*E. coli*
H31.1	Positive	Negative	Negative	Positive	*E. coli*
H31.2	Positive	Negative	Negative	Positive	*E. coli*
H31.3	Positive	Negative	Negative	Positive	*E. coli*
H32.1	Positive	Negative	Negative	Positive	*E. coli*
H32.2	Positive	Negative	Negative	Positive	*E. coli*
H32.3	Positive	Negative	Negative	Positive	*E. coli*
H33.1	Positive	Negative	Negative	Positive	*E. coli*
H33.2	Positive	Negative	Negative	Positive	*E. coli*
H33.3	Positive	Negative	Negative	Positive	*E. coli*
H34.1	Positive	Negative	Negative	Positive	*E. coli*
H34.2	Positive	Negative	Negative	Positive	*E. coli*
H34.3	Positive	Negative	Negative	Positive	*E. coli*
H35.1	Positive	Negative	Negative	Positive	*E. coli*
H35.2	Positive	Negative	Negative	Positive	*E. coli*
H35.3	Positive	Negative	Negative	Positive	*E. coli*
H36.1	Positive	Negative	Negative	Positive	*E. coli*
H36.2	Positive	Negative	Negative	Positive	*E. coli*
H36.3	Positive	Negative	Negative	Positive	*E. coli*
H37.1	Positive	Negative	Negative	Positive	*E. coli*
H37.2	Positive	Negative	Negative	Positive	*E. coli*
H37.3	Positive	Negative	Negative	Positive	*E. coli*
H38.1	Positive	Negative	Negative	Positive	*E. coli*
H38.2	Positive	Negative	Negative	Positive	*E. coli*
H38.3	Positive	Negative	Negative	Positive	*E. coli*
H39.1	Positive	Negative	Negative	Positive	*E. coli*
H39.2	Positive	Negative	Negative	Positive	*E. coli*
H39.3	Positive	Negative	Negative	Positive	*E. coli*

Table 4.11: Biochemical tests (catalase, urease, oxidase, and triple sugar iron) were used to identify bacteria in soil samples.

Sample No	Catalase	Urease	Oxidase	Triple sugar iron agar	Presumptive bacteria
S 1.1	Positive	Negative	Negative	Positive	*E. coli*
S 1.2	Positive	Negative	Negative	Positive	*E. coli*
S1.3	Positive	Negative	Negative	Positive	*E. coli*
S2.1	Positive	Negative	Negative	Positive	*E. coli*
S2.2	Positive	Negative	Negative	Positive	*E. coli*
S2.3	Positive	Negative	Negative	Positive	*E. coli*
S3.1	Positive	Negative	Negative	Positive	*E. coli*
S3.2	Positive	Negative	Negative	Positive	*E. coli*
S3.3	Positive	Negative	Negative	Positive	*E. coli*
S4.1	Positive	Negative	Negative	Positive	*E. coli*
S4.2	Positive	Negative	Negative	Positive	*E. coli*
S4.3	Positive	Negative	Negative	Positive	*E. coli*
S5.1	Positive	Negative	Negative	Positive	*E. coli*
S5.2	Positive	Negative	Negative	Positive	*E. coli*
S5.3	Positive	Negative	Negative	Positive	*E. coli*
S6.1	Positive	Negative	Negative	Positive	*E. coli*
S6.2	Positive	Negative	Negative	Positive	*E. coli*
S6.3	Positive	Negative	Negative	Positive	*E. coli*
S7.1	Positive	Negative	Negative	Positive	*E. coli*
S7.2	Positive	Negative	Negative	Positive	*E. coli*
S7.3	Positive	Negative	Negative	Positive	*E. coli*
S8.1	Positive	Negative	Negative	Positive	*E. coli*
S8.2	Positive	Negative	Negative	Positive	*E. coli*
S8.3	Positive	Negative	Negative	Positive	*E. coli*
S9.1	Positive	Negative	Negative	Positive	*E. coli*
S9.2	Positive	Negative	Negative	Positive	*E. coli*
S9.3	Positive	Negative	Negative	Positive	*E. coli*
S10.1	Positive	Negative	Negative	Positive	*E. coli*
S10.2	Positive	Negative	Negative	Positive	*E. coli*
S10.3	Positive	Negative	Negative	Positive	*E. coli*
S11.1	Positive	Negative	Negative	Positive	*E. coli*
S11.2	Positive	Negative	Negative	Positive	*E. coli*
S11.3	Positive	Negative	Negative	Positive	*E. coli*
S12.1	Positive	Negative	Negative	Positive	*E. coli*
S12.2	Positive	Negative	Negative	Positive	*E. coli*
S12.3	Positive	Negative	Negative	Positive	*E. coli*
S13.1	Positive	Negative	Negative	Positive	*E. coli*

S13.2	Positive	Negative	Negative	Positive	*E. coli*
S13.3	Positive	Negative	Negative	Positive	*E. coli*
S14.1	Positive	Negative	Negative	Positive	*E. coli*
S14.2	Positive	Negative	Negative	Positive	*E. coli*
S14.3	Positive	Negative	Negative	Positive	*E. coli*
S15.1	Positive	Negative	Negative	Positive	*E. coli*

S15.2	Positive	Negative	Negative	Positive	*E. coli*
S15.3	Positive	Negative	Negative	Positive	*E. coli*
S16.1	Positive	Negative	Negative	Positive	*E. coli*
S16.2	Positive	Negative	Negative	Positive	*E. coli*
S16.3	Positive	Negative	Negative	Positive	*E. coli*
S17.1	Positive	Negative	Negative	Positive	*E. coli*
S17.2	Positive	Negative	Negative	Positive	*E. coli*
S17.3	Positive	Negative	Negative	Positive	*E. coli*
S18.1	Positive	Negative	Negative	Positive	*E. coli*
S18.2	Positive	Negative	Negative	Positive	*E. coli*
S18.3	Positive	Negative	Negative	Positive	*E. coli*
S19.1	Positive	Negative	Negative	Positive	*E. coli*
S19.2	Positive	Negative	Negative	Positive	*E. coli*
S19.3	Positive	Negative	Negative	Positive	*E. coli*
S20.1	Positive	Negative	Negative	Positive	*E. coli*
S20.2	Positive	Negative	Negative	Positive	*E. coli*
S20.3	Positive	Negative	Negative	Positive	*E. coli*
S21.1	Positive	Negative	Negative	Positive	*E. coli*
S21.2	Positive	Negative	Negative	Positive	*E. coli*
S21.3	Positive	Negative	Negative	Positive	*E. coli*
S22.1	Positive	Negative	Negative	Positive	*E. coli*
S22.2	Positive	Negative	Negative	Positive	*E. coli*
S22.3	Positive	Negative	Negative	Positive	*E. coli*
S23.1	Positive	Negative	Negative	Positive	*E. coli*
S23.2	Positive	Negative	Negative	Positive	*E. coli*
S23.3	Positive	Negative	Negative	Positive	*E. coli*
S24.1	Positive	Negative	Negative	Positive	*E. coli*
S24.2	Positive	Negative	Negative	Positive	*E. coli*
S24.3	Positive	Negative	Negative	Positive	*E. coli*
S25.1	Positive	Negative	Negative	Positive	*E. coli*
S25.2	Positive	Negative	Negative	Positive	*E. coli*
S25.3	Positive	Negative	Negative	Positive	*E. coli*
S26.1	Positive	Negative	Negative	Positive	*E. coli*
S26.2	Positive	Negative	Negative	Positive	*E. coli*
S26.3	Positive	Negative	Negative	Positive	*E. coli*

S27.1	Positive	Negative	Negative	Positive	*E. coli*
S27.2	Positive	Negative	Negative	Positive	*E. coli*
S27.3	Positive	Negative	Negative	Positive	*E. coli*
S28.1	Positive	Negative	Negative	Positive	*E. coli*
S28.2	Positive	Negative	Negative	Positive	*E. coli*
S28.3	Positive	Negative	Negative	Positive	*E. coli*
S29.1	Positive	Negative	Negative	Positive	*E. coli*
S29.2	Positive	Negative	Negative	Positive	*E. coli*
S29.3	Positive	Negative	Negative	Positive	*E. coli*
S30.1	Positive	Negative	Negative	Positive	*E. coli*
S30.2	Positive	Negative	Negative	Positive	*E. coli*
S30.3	Positive	Negative	Negative	Positive	*E. coli*
S31.1	Positive	Negative	Negative	Positive	*E. coli*
S31.2	Positive	Negative	Negative	Positive	*E. coli*
S31.3	Positive	Negative	Negative	Positive	*E. coli*
S32.1	Positive	Negative	Negative	Positive	*E. coli*
S32.2	Positive	Negative	Negative	Positive	*E. coli*
S32.3	Positive	Negative	Negative	Positive	*E. coli*
S33.1	Positive	Negative	Negative	Positive	*E. coli*
S33.2	Positive	Negative	Negative	Positive	*E. coli*
S33.3	Positive	Negative	Negative	Positive	*E. coli*

APPENDIX B: MULTI-DRUG RESISTANCE

Multi-drug resistance of *E. coli* in stool samples**.**

Antibiotic combination	**Number of *E. coli* isolates**
Resistance to two antibiotics)	**n=%**
FOX + CIP	1 (0.85%)
ATH + AP	1 (0.85%)
AP + CIP	1 (0.85%
ATH + FOX	1 (0.85%)
ATH + NA	2 (1.71%)
AP + T	1 (0.85%)
AP +CL	4 (3.42%)
AP + C	3 (3.42%)
AK + ATH	1 (0.85%)
C + NA	2 (1.71%)
C + CTX	2 (1.71%)
C+CL	1 (0.85%)
A+C	1 (0.85%)
FOX +T	1 (0.85%)

AP+CTX	1 (0.85%)
A+T	1 (0.85%)
C+A	3 (3.42%)
C+FOX	1 (0.85%)
FOX+GM	1 (0.85%)
AP+AK	1 (0.85%)
CIP+AK	1 (0.85%)
CIP+C	1 (0.85%)
AP+ A	1 (0.85%)
CTX+A	1 (0.85%)
CTX+ATH	1 (0.85%)
C+AK	1 (0.85%)
NA+A	1 (0.85%)
ATH+A	1 (0.85%)
NA+C	2 (1.71%)
Total	**40 (34.18%)**
Resistance to three or more antibiotics)	
CL+CTX+FOX	1 (0.85%)
CTX+AP+C	1 (0.85%)
ATH+CL+A	1 (0.85%)
FOX+C+CIP	1 (0.85%)
CIP+CL+ATH	1 (0.85%)
ATH+C+AP	1 (0.85%)
CL+CTX+A	1 (0.85%)
NA+AK+C	2 (1.71%)
C+AK+A	1 (0.85%)
GM+ATH+CL	1 (0.85%)
AP+T+C	1 (0.85%)
NA+CIP+C	1 (0.85%)
FOX+AP+ATH	1 (0.85%)
ATM+C+AP	1 (0.85%)
ATH+A+CTX	1 (0.85%)
AK+C+CTX	1 (0.85%)
AK+C+FOX	1 (0.85%)
C+T+NA+CL	1 (0.85%)
NA+AP+FOX	1 (0.85%)
FOX+CTX+AP+CIP+A+NA+CL+ATH	1 (0.85%)
FOX+CTX+AP+CL	1 (0.85%)
FOX+CTX+AP+C+A+NA+CL	1 (0.85%)
FOX+CTX+AP+C+A+CL	1 (0.85%)
FOX+CTX+C+A+CL	1 (0.85%)
CTX+AP+A+NA+CL	1 (0.85%)

AP+C+A+CL	1 (0.85%)
CTX+AK+T+ATH	1 (0.85%)
CTX+AP+A+NA+CL+ATH	1(0.85%)
ATH+ATM+NA+A+C+GM+AP+CTX	1 (0.85%)
ATH+CL+C+A	1 (0.85%)
FOX+AK+A+CL	1 (0.85%)
ATH+A+AP+CTX+FOX	1 (0.85%)
CTX+C+T+CL	1 (0.85%)
A+T+AK+C	1 (0.85%)
FOX+CIP+C+CL	1 (0.85%)
FOX+AP+C+A+CL	1 (0.85%)
CTX+AP+C+A+CL	1 (0.85%)
AP+GM+C+CIP	1 (0.85%)
FOX+AP+C+CL	1 (0.85%)
CTX+A+NA+CL	1 (0.85%)
FOX+C+T+A+NA	1 (0.85%)
GM+NA+CL+ATH	1 (0.85%)
CTX+AP+CL+ATH	1 (0.85%)
CTX+AP+AK+NA+ATH	1 (0.85%)
FOX+CTX+ATH+CL+NA	1 (0.85%)
Total	**48 (41.02%)**
Overall total	**88 (75.20%)**

Keywords: AP= Ampicillin, A= Amoxycillin, T= Tetracycline, C=Chloramphenicol, ATH= Azithromycin, MEM= Meropenem, GM= Gentamycin, CL= Cephalexin, CTX-M= Cefotaxime, CIP= Ciprofloxacin, FOX= Cefoxitin, AK= Amikacin, ATM= Aztreonam and NA= Nalidixic acid.

Table 4.13: Multi-drug resistance of *E. coli* in soil samples**.**

Antibiotic resistance combination	**Number of *E. coli* isolates**
Resistance to two antibiotics	**n (%)**
CL+FOX	2 (2.12%)
CL+AK	1 (1.06%)
T+ATH	2 (2.12%)
CL+ATH	1 (1.06%)
AP+A	1 (1.06%)
ATH+C	2 (2.12%)
CL+C	3 (3.19%)
C+AK	1 (1.06%)
AK+A	1 (1.06%)
GM+C	1 (1.06%)
CTX+C	1 (1.06%)
C+T	1 (1.06%)
FOX+T	1 (1.06%)

FOX+ATH	1 (1.06%)
CTX+CL	1 (1.06%)
AP+C	1 (1.06%)
A+CL	1 (1.06%)
Total	**22 (23%)**
Resistance to three or more antibiotics	
FOX+CL+ATH	2 (2.12%)
FOX+C+CL	2 (2.12%)
AP+A+ATH	1 (1.06%)
CIP+CL+ATH	2 (2.12%)
ATH+C+A	2 (2.12%)
CTX+AP+ATH	1 (06%)
CTX+CL+ATH	2 (2.12%)
C+CIP+ATH	1 (1.06%)
GM+A+CL	1 (1.06%)
CTX+C+ATH	1 (1.06%)
FOX+C+ATH	1 (1.06%)
FOX+C+GM	1 (1.06%)
FOX+T+ATH	1 (1.06%)
C+CL+ATH	1 (1.06%)
FOX+T+CL	1 (1.06%)
CTX+CIP+AP	1 (1.06%)
FOX+CTX+A+NA	1 (1.06%)
GM+C+CL+ATH	1 (1.06%)
FOX+CTX+A+C	1 (1.06%)
C+AP+CIP+A	1 (1.06%)
FOX+C+AK+T	1 (1.06%)
CTX+CIP+T+NA+ATH	1 (1.06%)
CTX+GM+C+T+ATH	1 (1.06%)
A+C+GM+AP	1 (1.06%)
ATM+C+GM+CTX+FOX	1 (1.06%)
NA+AK+C+GM+AP+FOX	1 (1.06%)
FOX+AP+C+CIP+A+ATH	1 (1.06%)
FOX+CTX+C+CIP+T+A+CL	1 (1.06%)
FOX+CTX+C+CIP+A+ATH	1 (1.06%)
CTX+A+NA+ATM+FOX	1 (1.06%)
FOX+CTX+AP+C+A+ATM+ATH	1 (1.06%)
FOX+CTX+C+CIP+NA	1 (1.06%)
FOX+CTX+AK+A+CIP+C	1 (1.06%)
CTX+C+A+ATH	1(1.06%)
C+CIP+T+CL+A	1 (1.06%)
GM+C+NA+ATH	1 (1.06%)

C+AP+A+ATH	1 (1.06%)
FOX+AP+A+CL+ATH	1 (1.06%)
FOX+C+CL+ATH	1 (1.06%)
AP+A+CL+ATH	1 (1.06%)
CL+C+A+ATH	1 (1.06%)
FOX+CTX+GM+CL	1 (1.06%)
FOX+CL+A+T+AP	2 (2.12%)
FOX+AP+C+CL	1 (1.06%)
FOX+C+AK+A+CL	1 (1.06%)
Total	**61 (65%)**
Overall total	**83 (88%)**

Keywords: AP= Ampicillin, A= Amoxycillin, T= Tetracycline, C=Chloramphenicol, ATH= Azithromycin, MEM= Meropenem, GM= Gentamycin, CL= Cephalexin, CTX-M= Cefotaxime, CIP= Ciprofloxacin, FOX= Cefoxitin, AK= Amikacin, ATM= Aztreonam and NA= Nalidixic acid.