PREVALENCE OF COLISTIN RESISTANT ENTEROBACTERALES ISOLATED FROM POULTRY COMPOSTED MANURE IN BENIN CITY, NIGERIA.

BY

EKPENISI DEBORAH

BMS1802446

DEPARTMENT OF MEDICAL LABORATORY SCIENCE,
SCHOOL OF BASIC MEDICAL SCIENCE,
COLLEGE OF MEDICAL SCIENCES,
UNIVERSITY OF BENIN,
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A PROJECT SUBMITTED TO THE
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PROF. (MRS). H. O. OGEFERE

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CERTIFICATION

This is to certify that this research work will be carried out by **EKPENISI DEBORAH** with Matriculation Number **BMS1802446** under the supervision of **PROF. (MRS). H. O. OGEFERE** in the Department of Medical Laboratory Sciences, University of Benin, Benin city, Edo State.

PROF. (MRS.) H. O. OGEFERE **Project Supervisor**	**DATE**
DR. (MRS.) Z. OMORUYI **Head Of Department**	**DATE**
External Examiner	**DATE**

DEDICATION

This work is dedicated to Almighty God and my parents Mr and Mrs. WILLIAMS ISIMAH OGOCHUKWU

ACKNOWLEDGMENT

All praise, thanks and adoration be to God, the father of mercy who has given me the strength, wisdom, knowledge and understanding of all challenges to commence and conclude this project successfully.

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ABSTRACT

Colistin, a "last-resort" antibiotic for multidrug-resistant Gram-negative bacterial infections, has seen increasing use, leading to the emergence of colistin-resistant strains. The primary aim of this study was to investigate the prevalence of colistin-resistant Enterobacterales in composted manure samples collected from different farms in Benin City, Nigeria. This study was carried out in some selected poultry farms within Benin City, Nigeria. A cross-sectional study which involved collecting composted manure samples from each selected site using sterile containers. Samples were homogenized in buffered peptone water within 24 hours post collection from the farm. The composted manure were cultured on Mac-Conkey agar plate and incubated at 37^0c for 18-24 hours. Isolates were identified based on colonial morphology, motility, lactose fermentation, Gram staining reaction and biochemical tests (indole, citrate, oxidase and urease tests) The antimicrobial susceptibility testing was performed using kirby-Bauer disc diffusion method. Broth macrodilution was used to determine minimum inhibitory concentration (MIC) of colistin in calcium enhanced mueller-Hinton broth ($MIC \leq 2$). A total of 11 enterobacterales were isolated from 272 compost manure samples, consisting of 5 isolate of Escherichia coli, 3 isolates of klebsiella species, 2 isolate of proteus species and 1 isolate of *providencia spp.* Of the 5 *E. coli* samples isolated, 1(20%) showed collistin resistance. Of the 3 *Klebsiella spp isolated* none showed resistance to colistin. 2 Proteus samples were isolated, all of which showed resistance to colistin. Only one isolate of Providencia was found and showed resistance to colistin in the single isolate found. The total prevalence of colistin resistance in isolated enterobacterales was 4/11(36.4%). This study revealed a concerning prevalence of colistin resistance among Enterobacterales isolated from composted manure in Benin City, Nigeria, with resistance observed in key pathogens including *E. coli, Proteus spp* and *Providencia spp*. These findings highlight the critical role of environmental reservoirs in the spread of antibiotic resistance and underscore the potential public health implications, particularly in the context of last-resort treatments for multidrug-resistant infections.

CHAPTER ONE

INTRODUCTION

1.1.Background Of Study

Antimicrobial resitance (AMR) is a rapidly growing worldwide health concern that threatens the entire foundation of contemporary medicine. This process occurs when bacteria, viruses, parasites, and fungus develop methods to withstand the medications used to remove them. As a result, the efficacy of these antimicrobial drugs is gradually decreasing, leaving humans vulnerable to formerly treatable illnesses (Broom *et al*., 2021). AMR is a very complicated problem that affects many parts of healthcare. It is caused by overuse and abuse of antimicrobial drugs. Inadequate infection prevention and control strategies in healthcare Sttings exacerbate the problem, contributing to the development of drug-resistant organisms (Chandler, 2019). Antimicrobial resistance has serious repercussions, including longer illnesses, higher mortality rates, rising healthcare expenditures, and the possibility of previously curable diseases becoming untreatable (Walia, 2003). According to recent worldwide estimates, the disease burden of AMR is at least equal to that of HIV and malaria combined, with a projected 4.95 million deaths in 2019 (Elton *et al*., 2020). If not handled effectively, AMR might kill 10 million people each year and cost the world economy up to $100 trillion by 2050 (Fong, 2023). In 2019, it was estimated that the highest rates of AMR burden were in sub-Saharan Africa, among all regions (Murray *et al*., 2022). However, in sub-Saharan Africa, inadequate surveillance and regulation make it challenging to define the accurate status of AMR and to implement effective and sustainable programmes to address it (Masich *et al*., 2020).

The emergence of multidrug resistance defined as an organism showing resistance to three or more classes of antibiotics in enterobacteriales is particularly concerning (Park, 2018). The commonest examples of these are extended-spectrum β-lactamases, carbapenemases and for colistin, the presence of the mcr-1 gene (Bastidas *et al*., 2022).

Colistin is an antimicrobial agent extracted from *Paenibacillus polymyxa,* which comes under the class polymyxin group. Class polymyxin antibiotic contains five polymyxins: A, B, C, D, and E, of which polymyxin E (colistin) and polymyxin B are clinically relevant (Poirel *et al*., 2017). In humans, colistin sulfate (CS) is used for oral and topical administration, while colistin methane sulfonate (CMS) sodium is used for parenteral treatment (Rhouma *et al*., 2016).

Recent research emphasised the rising threat of colistin resistance mediated by mcr genes, with recorded cases found in animals, people, food, and the environment (Elbediwi *et al*., 2019). Colistin is one of the last resort treatments for multidrug-resistant Gram-negative bacterial infections (Anyanwu *et al*., 2023). Colistin is widely used in animal husbandry for treatment and as a growth stimulant in food supplements, either at normal or excessively high levels, enhancing the selection for colistin resistance in animals (Poirel *et al*., 2017). Furthermore, antibiotics, including colistin, are losing potency due to their overuse in veterinary treatment. Indeed, in certain nations, the usage of colistin was 600 times higher in animals than in people. (Skov and Monnet, 2016).

In 2016, Liu *et al.* discovered for the first time in China a plasmid-mediated colistin-resistance gene, a mobile element containing the mcr-1 gene. This gene encodes the production of phosphoethanolamine transferase, which affects lipid A and imparts antibiotic resistance in Enterobacteriales (Liu *et al*., 2016). Colistin-resistant Enterobacteriales, mainly *Escherichia coli* and *Klebsiella pneumoniae*, have been observed globally, including in the Americas (Bastidas *et al*., 2022). According to studies conducted in Latin America, this expansion might be linked to the horizontal transmission of colistin-resistant genes (Hoang *et* *al*., 2022). Since the identification of the mcr-1 gene and its presence in Enterobacteriales plasmids, global surveillance of colistin resistance has increased (Poirel *et al*., 2017).

Also in 2016, the first clinical isolate of colistin-resistant *Escherichia coli* harbouring the mcr-1 gene was reported in an adolescent with appendicitis (Ortega *et al*., 2016). Since then, studies of animals on rural farms where there is extensive use of colistin as a growth promoter have shown widespread distribution of colistin resistant *E. coli*, with a high proportion of isolates containing mcr-1 genes (Montero *et al*., 2021; Yamamoto *et al*., 2019). The reported prevalence of colistin-resistant Escherichia coli in animals from farms employing colistin as a growth promoter further emphasizes the need to explore the role of composted manure in facilitating the dissemination of antibiotic resistance. Understanding the prevalence in composted manure is crucial as it forms a potential link between agricultural practices and the broader dissemination of colistin resistance, impacting both environmental and public health.

1.2. Statement Of Problem

The presence of colistin-resistant Enterobacteriales in composted manure raises serious public health and environmental concerns. Because colistin is an important last-resort antibiotic against multidrug-resistant Gram-negative bacterial infections, the establishment of resistance in Enterobacteriales raises concerns about its potential consequences for human health and agricultural operations. Given the extensive use of colistin in animal husbandry and as a growth stimulant in food supplements, there is an urgent need to research the level of colistin resistance in Enterobacteriales isolated from composted manure. Understanding the incidence of resistance in this particular environment is crucial for determining the role of composted manure in the spread of antibiotic resistance, as well as bridging the gap between agricultural practices and the larger public health consequences of antibiotic resistance.

1.3. Justification Of Study

This research is justified by the pressing need to comprehensively understand and address the potential environmental dissemination of antibiotic resistance, particularly in the context of agricultural practices. Colistin, a critically important antibiotic for treating multidrug-resistant Gram-negative bacterial infections in humans, is increasingly facing challenges due to the emergence and spread of resistance, notably mediated by the mcr genes. Also, antibiotic-resistant Enterobacterales pose a direct threat to public health. If composted manure is identified as a source of colistin-resistant strains, it becomes essential to evaluate the potential pathways through which these bacteria may reach humans, either through direct contact with contaminated soil or water, or through the food chain.

1.4 Aim of Study

The aim of this study is to determine the prevalence of colistin resistant enterobacteriales isolated from poultry composted manure in Benin city, Nigeria.

1.5 Specific Objectives

Specific Objectives are:

1. to Isolate and identify specific Enterobacterales bacteria in composted manure samples in Benin City, Nigeria.
2. To determine the prevalence of colistin resistant enterobacterales isolated from composted manure in Benin city, Nigeria.

1.6 Research Questions

1. What are the specific enterobacterales present in composted manure samples in Benin City, Nigeria?

2. What is the prevalence of colistin-resistant enterobacterales among the isolated bacteria in composted manure collected in Benin City, Nigeria?

1.7 Research Hypothesis

Null hypothesis

There is low prevalence of colistin resistant enterobacteriales isolated from composted manure in Benin city, Nigeria.

Alternate hypothesis

There is high prevalence of colistin resistant enterobacteriales isolated from composted manure in Benin City, Nigeria.

CHAPTER TWO

LITERATURE REVIEW

2.1.1 Review of Colistin

Colistin, known scientifically as polymyxin E, is a potent antibiotic that falls under the polymyxin class of antimicrobial agents. This antibiotic has a critical role in contemporary medicine, especially as a last-resort treatment against infections caused by Gram-negative bacteria that are resistant to a broad range of antibiotics (Bialvaei *et al*., 2015). It's particularly effective against formidable pathogens such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and certain strains of *Escherichia coli*, which are known for their resilience against multiple drugs (Ordooei *et al*., 2015).

Discovered in 1949 from the bacterium Paenibacillus polymyxa var. colistinus, colistin was among the earlier antibiotics used to fight bacterial infections. Its usage was widespread during its early years of discovery due to its effectiveness. However, the initial enthusiasm for colistin waned by the 1970s because of growing concerns over its potential to cause significant nephrotoxicity (kidney damage) and neurotoxicity (damage to the nervous system), leading to a decrease in its clinical application. With the turn of the century and the alarming rise in antibiotic resistance, colistin has re-emerged as a crucial element in the arsenal against multidrug-resistant bacterial infections, highlighting the cyclic nature of antibiotic utilization based on emerging healthcare needs and understanding (El-Sayed *et al*., 2020). The mechanism through which colistin combats bacteria is both fascinating and brutal. Colistin targets the lipid A component of lipopolysaccharides (LPS) in the outer membrane of Gram-negative bacteria. By binding to these components, colistin disrupts the integrity of the bacterial cell membrane, causing leakage of cellular contents and ultimately leading to bacterial cell death. This mode of action is particularly effective against bacteria that have developed resistance to other antibiotics, making colistin a valuable resource in treating severe infections.

Colistin is administered in two main forms: colistin methanesulfonate (CMS) for intravenous use and colistin sulfate for topical application or oral administration. CMS, a prodrug of colistin, is converted into the active form in the body and is primarily used for severe systemic infections (Gharaibeh and Shatnawi, 2019). Colistin sulfate, on the other hand, is used to treat infections in the gastrointestinal tract and as a topical agent for wound infections. Despite its efficacy, the use of colistin is tempered by concerns over its side effects, particularly its impact on kidney function and the nervous system, necessitating careful patient monitoring (Hamel *et al*., 2021).

The resurgence of colistin as a critical therapeutic option underscores the pressing need for novel antibiotics and strategies to combat antibiotic-resistant bacteria. While colistin plays a vital role in managing difficult infections, the emergence of colistin-resistant bacteria presents a significant challenge, emphasizing the importance of antimicrobial stewardship and the ongoing search for new antimicrobial agents. The situation with colistin is a stark reminder of the dynamic interplay between microbial evolution and human innovation in the realm of infectious disease management (Velkov *et al*., 2021).

2.1.2 Discovery and Historical Use

Colistin was discovered in 1949, a time marked by fervent activity in antibiotic research following the revolutionary introduction of penicillin. The discovery came from the bacterium Paenibacillus polymyxa var. colistinus, which produced this potent compound naturally as a defense mechanism against competing microbial species (Hamel *et al*., 2021). The identification of colistin as an effective agent against Gram-negative bacteria was a significant achievement, given the limited options available at the time for treating infections caused by these organisms (Bialvaei *et al*., 2015).

Following its discovery, colistin quickly found its way into clinical use. During the 1950s and 1960s, it became widely adopted for treating infections caused by Gram-negative bacteria, including *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Escherichia coli*. These pathogens are known for causing severe and often life-threatening infections, such as pneumonia, urinary tract infections, and bacteremia. The ability of colistin to combat these bacteria made it a valuable asset in the medical community's arsenal against infectious diseases.

Despite its early success, the use of colistin began to wane by the 1970s. This decline was primarily due to growing concerns over its potential side effects, including nephrotoxicity (kidney damage) and neurotoxicity (damage to the nervous system). These side effects were significant enough to prompt the medical community to search for safer alternatives, leading to a decrease in the clinical application of colistin (Velkov *et al*., 2021). The development of newer antibiotics during this period also contributed to colistin being sidelined, as these alternatives were perceived to be equally effective but with a lower risk of adverse effects.

The turn of the 21st century marked a significant shift in the landscape of infectious disease treatment. The rising tide of antibiotic resistance, particularly among Gram-negative bacteria, posed a formidable challenge to public health (Velkov *et al*., 2021). Many of the antibiotics developed in the latter half of the 20th century were becoming less effective due to the widespread emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) bacterial strains. This crisis led to the revaluation of colistin's role in clinical medicine.

Recognizing its potency against resistant bacteria, colistin was reintroduced into the therapeutic arsenal, this time as a "last-resort" antibiotic for treating serious infections that were unresponsive to other treatments (Bialvaei *et al*., 2015). The resurgence of colistin reflects the dynamic nature of antibiotic use, where drugs can fall out of favour due to concerns over safety or efficacy but may later regain prominence as circumstances change.

Historical Impact and Lessons Learned

The history of colistin underscores several important themes in the field of infectious disease treatment. First, it highlights the constant arms race between bacterial evolution and antibiotic development, illustrating how bacteria can rapidly adapt to overcome therapeutic interventions. Second, it serves as a reminder of the critical need for judicious antibiotic use to minimize the development of resistance. Finally, colistin's story emphasizes the importance of ongoing research and development in the quest for new and safer antibiotics to address the ever-evolving challenge of bacterial infections (Hamel *et al*., 2021).

As we move forward, the lessons learned from colistin's history will undoubtedly continue to inform our approach to managing infectious diseases, emphasizing the delicate balance between leveraging the tools at our disposal and preserving their efficacy for future generations.

2.2.1 Mechanism of Action

The mechanism of action of colistin, a potent antibiotic belonging to the polymyxin class, is a fascinating example of how certain antibiotics exert their effects on bacteria, specifically targeting Gram-negative organisms. Colistin’s mode of action is primarily bactericidal, meaning it kills bacteria rather than merely inhibiting their growth. This effect is achieved through a multi-step process that disrupts the integrity of the bacterial cell membrane, leading to cell death (Bialvaei *et al.*, 2015).

Targeting the Bacterial Cell Membrane

1. **Interaction with Lipopolysaccharides (LPS):** The outer membrane of Gram-negative bacteria is composed of a complex molecule known as lipopolysaccharide (LPS). LPS plays a crucial role in maintaining the structural integrity of the bacterial cell membrane and in protecting the bacterium from harmful substances. Colistin specifically targets the lipid, A component of LPS (Andrade *et al.*, 2020). Lipid A is a critical part of the LPS molecule that helps anchor LPS in the outer membrane.

2. **Disruption of the Membrane Structure:** Colistin binds to the phosphate groups of lipid A, which disrupts the LPS layer and compromises the outer membrane's integrity. This binding destabilizes the membrane by displacing divalent cations (Ca^{2+} and Mg^{2+}) that normally stabilize the LPS molecules (Gogry *et al.*, 2021). The loss of these cations leads to an increase in membrane permeability.

Leakage and Cell Death

1. **Increased Permeability:** As the outer membrane becomes permeable, the bacterium is unable to control the flow of molecules across its membrane. This disruption affects the bacterial cell's ability to maintain its internal environment, leading to the leakage of vital cellular contents.

2. **Inner Membrane Disruption:** The damage inflicted by colistin is not limited to the outer membrane. Once the outer barrier is compromised, colistin can also interact with the inner membrane, further disrupting its structure and function. This interaction

exacerbates the leakage of cellular components and disrupts critical processes within the cell.

3. **Cell Death:** The combined effects of outer and inner membrane disruption lead to the loss of essential ions and molecules, disruption of metabolic processes, and eventual cell lysis (bursting of the cell). This culminates in the bactericidal effect of colistin, effectively killing the bacteria.

The mechanism of action of colistin is particularly effective against Gram-negative bacteria due to the unique composition of their outer membrane, which is rich in LPS. This selectivity is a key feature of colistin's antibacterial activity, as it spares Gram-positive bacteria and human cells, which lack the LPS structure. However, the toxic effects of colistin on human tissues, particularly the kidneys and nervous system, are a concern and limit its use to cases where other antibiotics are ineffective (Gogry *et al.*, 2021).

2.2.2 FORMULATIONS, DOSAGE, and ROUTE OF ADMINISTRATION

Colistin is available in two primary formulations, each suited to different therapeutic needs and routes of administration. These formulations include:

1. **Colistin Methanesulfonate (CMS):** Also known as colistimethate sodium, CMS is an inactive prodrug that is converted to the active form of colistin (colistin A and colistin B) in the body. This formulation is primarily used for parenteral administration, including intravenous (IV) injection or infusion, to treat severe systemic infections caused by multidrug-resistant Gram-negative bacteria (Feng *et al.*, 2021).

2. **Colistin Sulfate:** This formulation is used for topical applications, such as in wound infections, and for oral administration to target infections in the gastrointestinal tract.

When given orally, colistin sulfate is not significantly absorbed into the bloodstream, making it effective for treating localized infections within the gut without systemic exposure (Feng *et al*., 2021).

Dosage and Route of Administration

The dosage of colistin and the chosen route of administration depend on various factors, including the severity of the infection, the site of infection, the patient's renal function, and the susceptibility of the infecting organism.

1. **Intravenous Colistin (CMS):** For systemic infections, CMS is administered intravenously. The dosage must be carefully adjusted based on the patient's renal function to minimize the risk of nephrotoxicity. The typical adult dose for treating severe infections is initiated with a loading dose, followed by maintenance doses that are adjusted according to creatinine clearance rates to avoid accumulation and toxicity.
2. **Oral Colistin Sulfate:** Used primarily for the treatment of gastrointestinal infections, the oral dose of colistin sulfate is determined based on the severity of the infection and the patient's weight. This route ensures local action within the gastrointestinal tract without significant systemic absorption (Zhang *et al*., 2023).
3. **Topical Colistin Sulfate:** For topical applications, colistin sulfate is applied directly to the infected area in the form of a cream, ointment, or powder. The concentration and frequency of application are tailored to the specific needs of the infection being treated.

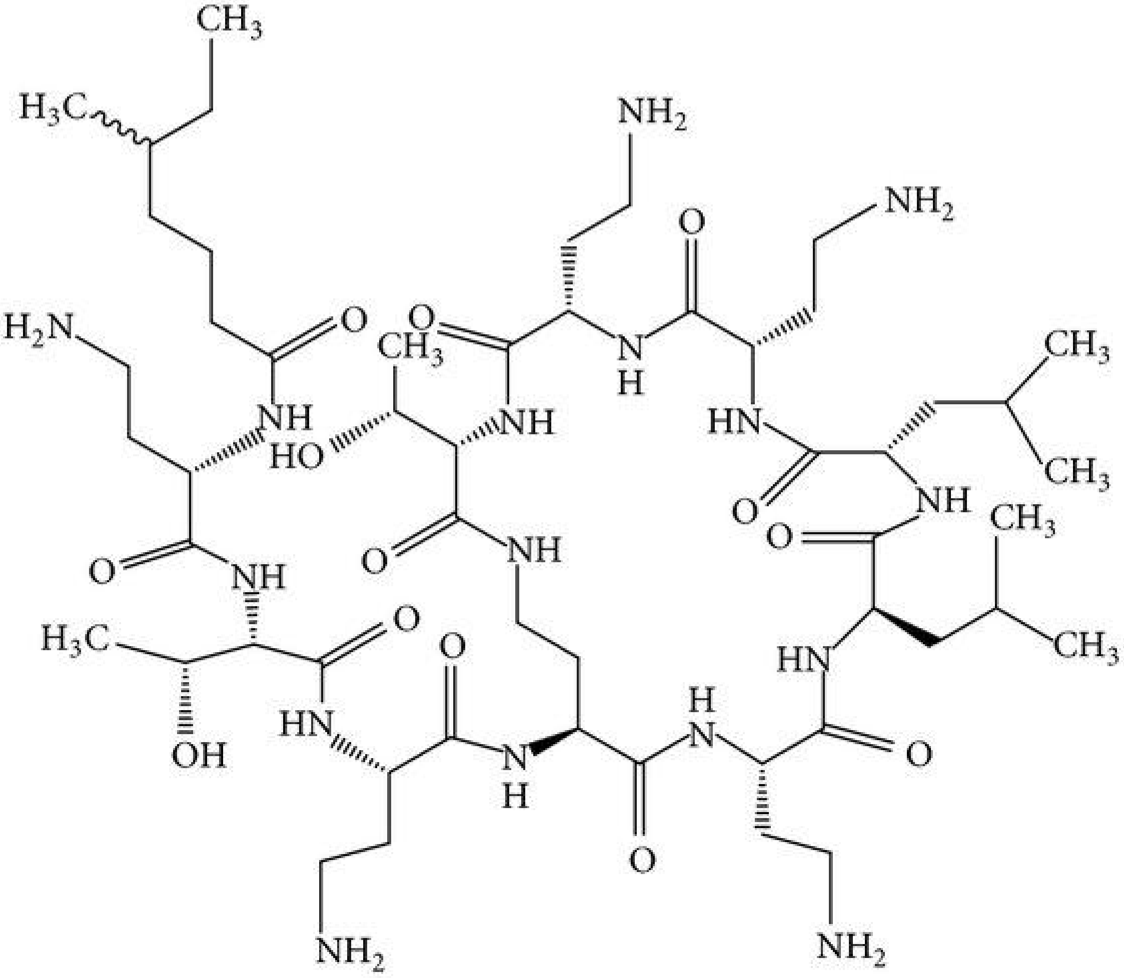

Figure 3.1 chemical structure of colistin (used as sulphate) (Bialvaei *et al*., 2015)

2.2.3 Indications for Use of Colistin

Indications

Colistin is indicated for the treatment of infections caused by multidrug-resistant Gram-negative bacteria, a growing concern in hospital settings and among immunocompromised patients. Specific indications include:

1. **Severe Respiratory Infections:** Including pneumonia caused by *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and other resistant bacteria.

2. **Urinary Tract Infections (UTIs):** Particularly those caused by carbapenem-resistant Enterobacteriaceae (Wacharachaisurapol *et al.*, 2020).

3. **Intra-abdominal Infections:** Such as peritonitis, especially in patients with limited treatment options due to antibiotic resistance.

4. **Gastrointestinal Infections:** Oral colistin sulfate is indicated for the treatment of gastrointestinal carriage of multidrug-resistant bacteria, aiming to reduce bacterial load and prevent systemic infection.

5. **Topical Use:** For the treatment of infected wounds, burns, and skin infections where multidrug-resistant Gram-negative bacteria are identified as the causative agents (Wacharachaisurapol *et al.*, 2020).

2.2.3 Toxicity

The use of colistin, particularly in the treatment of infections caused by multidrug-resistant Gram-negative bacteria, is associated with a significant risk of toxicity. This potential for adverse effects, especially nephrotoxicity (kidney damage) and neurotoxicity (damage to the nervous system), has been a major concern since its early clinical use and remains a critical factor in its deployment as a "last-resort" antibiotic. Understanding the nature and mechanisms of colistin's toxicity is essential for managing these risks and ensuring patient safety (Jafari and Elvasi, 2021).

Nephrotoxicity

Nephrotoxicity is the most commonly observed adverse effect associated with colistin use. The kidneys are particularly vulnerable to damage by colistin due to their role in concentrating and excreting the antibiotic. Colistin-induced nephrotoxicity can manifest as acute kidney injury (AKI), characterized by a rapid decline in renal function. This can result in various degrees of severity, ranging from increased serum creatinine and reduced urine output to more severe complications requiring dialysis (Long *et al.*, 2022). The mechanism underlying colistin's nephrotoxic effects is believed to involve direct toxicity to renal tubular cells, leading to cell death and tubular dysfunction. Factors that increase the risk of nephrotoxicity include higher doses, prolonged treatment duration, pre-existing kidney disease, and concomitant use of other nephrotoxic drugs (Dai *et al.*, 2022).

Neurotoxicity

Neurotoxic effects associated with colistin are less common than nephrotoxicity but can be severe. Symptoms of neurotoxicity may include dizziness, weakness, visual disturbances, confusion, and, in more severe cases, neuromuscular blockade leading to respiratory failure. The exact mechanism by which colistin induces neurotoxic effects is not fully understood but may involve disruption of neuronal cell membrane integrity and function. The risk of neurotoxicity is influenced by the dose of colistin, the route of administration, and the patient's overall health status (Bintang *et al.*, 2023).

Management of Colistin Toxicity

The management of colistin toxicity primarily involves careful dosing and monitoring of renal function. Adjusting the dose of colistin based on the patient's renal function and closely monitoring serum creatinine levels can help minimize the risk of nephrotoxicity (Wacharachaisurapol *et al*., 2020). For patients at high risk of toxicity or those who develop signs of kidney injury, dose reduction or discontinuation of colistin may be necessary. Supportive care, including hydration and, if necessary, renal replacement therapy, may be required for patients with significant nephrotoxicity (Bintang *et al*., 2023).

2.2.4 Synergy Between Colistin And Other Antibiotics

The concept of antibiotic synergy, where two or more antibiotics are used together to achieve a greater antimicrobial effect than would be expected from their individual effects, is an important strategy in combatting complex and multidrug-resistant bacterial infections (Jafari and Elvasi, 2021). Colistin, due to its unique mechanism of action and potent activity against Gram-negative bacteria, has been studied extensively for its potential synergistic interactions with other antibiotics. These combinations are particularly valuable in treating infections caused by pathogens resistant to multiple antibiotics, providing a critical advantage in clinical settings (Jafari and Elvasi, 2021).

Synergy with Carbapenems

One of the most studied combinations involves the use of colistin with carbapenems, a class of broad-spectrum beta-lactam antibiotics. Carbapenems are effective against a wide range of bacteria but have seen a rise in resistance, particularly among Gram-negative organisms. The synergistic effect of colistin and carbapenems is thought to arise from colistin’s ability to disrupt the bacterial outer membrane, enhancing the penetration of carbapenems into the bacterial cell and thereby increasing their bactericidal activity. This combination has shown promise in treating infections caused by carbapenem-resistant strains of Pseudomonas aeruginosa and Acinetobacter baumannii (Long *et al*., 2022).

Synergy with Rifampicin

Another notable combination is colistin with rifampicin, an antibiotic commonly used to treat tuberculosis and other bacterial infections. Rifampicin acts by inhibiting bacterial RNA synthesis, and when combined with colistin, has shown synergistic effects against various multidrug-resistant Gram-negative bacteria. This synergy is particularly effective against Acinetobacter baumannii, where the combination has been observed to significantly reduce bacterial counts and improve outcomes in infected animal models.

Synergy with Glycopeptides

The use of colistin in combination with glycopeptides, such as vancomycin, has also been explored, especially for treating infections caused by multidrug-resistant organisms. While glycopeptides primarily target Gram-positive bacteria, their combination with colistin can offer a broader antimicrobial coverage, encompassing tough-to-treat Gram-negative bacteria (Bialvaei *et al*., 2015). This synergy is thought to arise from the enhanced permeability of the bacterial cell membrane induced by colistin, allowing glycopeptides to access and disrupt the cell wall synthesis of Gram-negative bacteria more effectively.

Synergy with Fosfomycin

Colistin and fosfomycin constitute another combination with demonstrated synergy against multidrug-resistant Gram-negative bacteria. Fosfomycin inhibits an early step in bacterial cell wall synthesis and, when used in conjunction with colistin, has shown enhanced bactericidal activity. This combination has been particularly useful against Pseudomonas aeruginosa and

Klebsiella pneumoniae strains resistant to multiple antibiotics, offering a viable treatment option for severe infections (Bialvaei *et al*., 2015).

Mechanistic Basis and Clinical Implications

The synergistic effects observed between colistin and other antibiotics can be attributed to various mechanisms, including enhanced drug penetration, simultaneous targeting of multiple bacterial processes, and the suppression of resistance development. By employing multiple mechanisms of action, these combinations can effectively overcome the bacterial defenses and reduce the likelihood of resistance development.

2.3.1 Other Antibiotics of The Polymyxin Class

The polymyxin class of antibiotics, to which colistin (polymyxin E) belongs, includes several compounds characterized by their similar structure and mechanism of action, particularly their efficacy against Gram-negative bacteria. Polymyxin B is the other major antibiotic in this class that is used clinically. Like colistin, polymyxin B is utilized primarily as a last-resort treatment for infections caused by multidrug-resistant Gram-negative bacteria. Both antibiotics share a common mechanism—disrupting the bacterial cell membrane by binding to the lipid A component of lipopolysaccharides (LPS), leading to cell death (Dubashynskaya and Skorik, 2020) .

While colistin (polymyxin E) and polymyxin B are the most well-known and clinically significant members of the polymyxin class of antibiotics, there are other polymyxins that have been identified, including polymyxin A, C, D, and F. These antibiotics share a core structure and mechanism of action but differ in their fatty acid chains and peptide sequences, which can influence their antibacterial activity and toxicity profiles. The focus in clinical practice and research has predominantly been on colistin and polymyxin B due to their efficacy against multidrug-resistant Gram-negative bacteria. However, understanding the broader spectrum of polymyxins can provide insights into the potential for developing new antimicrobial strategies (Ontong *et al.*, 2020).

Polymyxin A

Polymyxin A is structurally similar to the other polymyxins but is less commonly used in clinical settings. Like colistin and polymyxin B, polymyxin A exhibits bactericidal activity against a range of Gram-negative bacteria by disrupting the bacterial cell membrane. However, its clinical use has been limited, partly due to concerns about toxicity and the availability of more effective or less toxic alternatives within the polymyxin class and other antibiotic classes.

Polymyxin C

Also known as polymyxin S, polymyxin C has been studied to a lesser extent compared to colistin and polymyxin B. It shares the general mechanism of action of polymyxins, targeting the lipid A component of lipopolysaccharides in the outer membrane of Gram-negative bacteria. The specific indications, efficacy, and safety profile of polymyxin C in clinical settings have not been as thoroughly explored or documented, leading to its limited use (Rodríguez-Santi *et al.*, 2021).

Polymyxin D

Polymyxin D, similar to the other polymyxins, has activity against Gram-negative bacteria through membrane disruption. Its utilization in medical practice has been minimal, and detailed studies on its pharmacokinetics, pharmacodynamics, and clinical applications are scarce. As with polymyxin A and C, the preference for colistin and polymyxin B in treating infections caused by multidrug-resistant Gram-negative bacteria has overshadowed the potential use of polymyxin D.

Polymyxin F

Polymyxin F, also known as mattacin, is another member of the polymyxin class. There is limited information available on polymyxin F, and its role in clinical medicine has not been established. Like its counterparts, it would be expected to have a similar mode of action involving the disruption of the bacterial cell membrane, but specific studies on its efficacy, safety, and potential clinical applications are lacking.

Potential for Future Development

Despite the focus on colistin and polymyxin B, the other polymyxins represent a reservoir of potential antimicrobial agents that could be further explored, especially in the face of rising antibiotic resistance. Advances in pharmaceutical technology and a better understanding of the mechanisms underlying toxicity could potentially unlock the therapeutic potential of these less-utilized polymyxins. For instance, modifications to the molecular structure of these antibiotics could enhance their safety profile or increase their efficacy against resistant bacteria (Haseeb *et al.*, 2021).

Moreover, the exploration of synergistic combinations involving lesser-known polymyxins with other antimicrobial agents could provide new avenues for treating complex infections. Such research would be critical in expanding the arsenal against multidrug-resistant organisms and addressing the global challenge of antibiotic resistance (Bintang *et al.*, 2021).

2.4 *Enterobacterales* – A Major Host For Colistin Resistance

Review of ***Enterobacterales***

1. Escherichia

Escherichia, with *Escherichia coli* as its flagship species, stands as a paradigm of bacterial diversity and adaptability, embodying the dual nature of bacteria as both vital contributors to life processes and formidable pathogens. This genus, positioned within the vast and medically significant order of *Enterobacterales*, comprises Gram-negative, facultatively anaerobic, rod-shaped bacteria that predominantly inhabit the gastrointestinal tract of humans and other warm-blooded animals. Escherichia's significance spans ecological, biological, and medical realms, reflecting its complex role in health and disease (Lee *et al*., 2022).

In the ecological and biological landscape, Escherichia species, particularly E. coli, are integral to the normal flora of the intestinal tract. They perform essential functions such as the breakdown of dietary lactose, synthesis of vital vitamins like vitamin K2, and formation of a barrier against pathogenic bacteria by competing for nutrients and attachment sites. This symbiotic relationship underscores the beneficial aspects of Escherichia, highlighting its role in maintaining gastrointestinal health and contributing to the host's immune defense mechanisms (Tompkins *et al*., 2021).

Pathogenic Potential

Despite the benign nature of most Escherichia strains, the genus is also notorious for its pathogenic members, which have adapted to exploit host vulnerabilities and cause disease. Pathogenic E. coli strains are categorized into various pathotypes based on their virulence properties, modes of transmission, and the clinical symptoms they elicit. These include:

- **Enteropathogenic E. coli (EPEC):** Known for causing diarrhea in infants, particularly in developing countries.
- **Enterotoxigenic E. coli (ETEC):** A leading cause of traveler's diarrhea and childhood diarrhea in low-income countries.
- **Enteroinvasive E. coli (EIEC):** Causes dysentery-like symptoms through the invasion and destruction of colonic epithelial cells.
- **Enterohemorrhagic E. coli (EHEC):** Associated with severe foodborne outbreaks, leading to bloody diarrhea and potentially life-threatening hemolytic uremic syndrome (HUS). The E. coli O157:H7 strain is the most infamous member of this group.
- **Enteroaggregative E. coli (EAEC):** Linked to persistent diarrhea in children and adults in both developed and developing countries.

Transmission and Disease Prevention

The transmission of pathogenic Escherichia strains primarily occurs through the fecal-oral route, facilitated by the consumption of contaminated food or water, direct contact with infected individuals, or exposure to contaminated environments. Preventive measures against E. coli infections hinge on food safety practices such as thorough cooking of meat, proper handling and washing of fruits and vegetables, and adherence to good hygiene practices including regular handwashing.

The issue of antibiotic resistance in Escherichia, particularly concerning Escherichia coli (E. coli), has become a pressing concern in the realm of infectious diseases. This challenge is epitomized by the emergence of resistance to colistin, a "last-resort" antibiotic primarily used for treating infections caused by multidrug-resistant Gram-negative bacteria. Colistin resistance in Escherichia species, notably E. coli, underscores the adaptive capacity of bacteria and presents a significant hurdle in the management of infectious diseases (Tompkins and van Duin, 2021).

Emergence of Colistin Resistance

Colistin has been a cornerstone in the treatment of severe infections caused by carbapenem-resistant Enterobacteriaceae, a category that includes some strains of E. coli. The discovery of colistin-resistant E. coli strains, particularly those carrying the plasmid-mediated mcr-1 gene, marked a pivotal moment in the ongoing battle against antibiotic resistance. The mcr-1 gene encodes a phosphoethanolamine transferase enzyme that modifies the lipid A component of lipopolysaccharides (LPS) on the bacterial outer membrane. This modification reduces the affinity of colistin for its binding targets, thereby conferring resistance to the bacterium (Binsker *et al.*, 2022).

The presence of colistin-resistant E. coli strains poses a grave challenge to public health, as it limits the therapeutic options available for treating severe and life-threatening infections. Infections caused by colistin-resistant E. coli are associated with higher rates of morbidity and mortality, particularly in hospital settings where multidrug-resistant organisms are more prevalent (Binsker *et al.*, 2022). The spread of colistin resistance further complicates the already daunting task of controlling antibiotic-resistant infections, necessitating stringent infection control measures, prudent antibiotic use, and the development of novel antimicrobial strategies (Touati and Mairi, 2020).

2. Salmonella

Salmonella, a significant genus within the order *Enterobacterales*, encompasses a complex group of Gram-negative bacteria known for their impact on human and animal health. This genus is intricately associated with both foodborne illnesses and systemic infections, illustrating a broad public health concern across the globe. Salmonella species are divided into two main species: *Salmonella bongori* and *Salmonella enterica*, with the latter being further divided into numerous serovars that have differing pathogenic profiles and host specificities (Bujňáková *et al.*, 2022).

Salmonella Pathogenesis

Salmonella enterica serovars, such as Typhimurium and Enteritidis, are among the most common causes of salmonellosis, a disease characterized by diarrhea, fever, and abdominal cramps. Transmission typically occurs through the ingestion of contaminated food or water, particularly from poultry, eggs, and meat, as well as through contact with infected animals or their environments (Bonnin *et al.*, 2021). The ability of Salmonella to infect a wide range of hosts, including humans, domestic animals, and wildlife, contributes to its widespread prevalence and the difficulty in controlling its spread (Patel *et al.*, 2020).

Upon ingestion, Salmonella bacteria can survive the acidic conditions of the stomach and then invade the epithelial cells of the small intestine. This invasion triggers an inflammatory response that disrupts the normal absorption of water and electrolytes, leading to diarrhea. Some serovars, such as Typhi and Paratyphi, are capable of causing more severe systemic infections, known as typhoid and paratyphoid fever, respectively. These infections can lead to high fever, severe headache, and even life-threatening complications if not treated promptly (Patel *et al.*, 2020).

Antibiotic Resistance in Salmonella

The emergence of antibiotic-resistant strains of Salmonella poses a significant challenge to the treatment of salmonellosis and systemic Salmonella infections. Resistance can occur through various mechanisms, including the acquisition of resistance genes on plasmids, mutations in chromosomal genes, and the expression of efflux pumps that expel antibiotics from the bacterial cell. Multidrug-resistant (MDR) strains, resistant to three or more classes of antibiotics, have been identified and are of particular concern, as they limit the options available for effective treatment (Bujňáková *et al.*, 2022).

The spread of antibiotic resistance among Salmonella serovars is facilitated by the use of antibiotics in animal husbandry, where they are used not only for treating infections but also as growth promoters (Camargo *et al.*, 2022). This practice selects for resistant bacteria, which can then be transmitted to humans through the food chain. The global dissemination of MDR Salmonella strains underscores the need for prudent use of antibiotics in both human medicine and agriculture.

Public Health Implications and Control Measures

Controlling Salmonella infections requires a multifaceted approach that includes surveillance of human and animal infections, rigorous food safety practices, and public education on proper food handling and hygiene. Measures such as cooking meat thoroughly, preventing cross-contamination in the kitchen, and washing hands regularly are effective in reducing the risk of Salmonella infection (Chen *et al.*, 2021).

At the regulatory level, monitoring the prevalence of Salmonella in food products and the environment, along with enforcing standards for food production and processing, are crucial for preventing outbreaks. Vaccination against Salmonella, particularly for serovars Typhi and

Paratyphi, is an important preventive measure in areas where typhoid fever is endemic (Lee *et al*., 2022).

The ongoing challenge of antibiotic resistance necessitates the development of novel antimicrobial agents and alternative strategies for controlling Salmonella infections. Research into bacterial pathogenesis, the host immune response, and the mechanisms of resistance is essential for identifying new targets for intervention and for designing effective public health strategies to combat this persistent threat (Tompkins van Duiin, 2021).

3. Shigella

Shigella spp.: Comprising several species, Shigella is a primary cause of bacillary dysentery or shigellosis, characterized by severe diarrhea, fever, and stomach cramps. Shigella is highly contagious and spreads via the fecal-oral route.

Shigella, a genus within the *Enterobacterales* order, is a key pathogen responsible for causing shigellosis, also known as bacillary dysentery, predominantly in humans (Bonnin *et al*., 2021). The genus is named after Kiyoshi Shiga, who first discovered the bacterium as the causative agent of a severe dysentery outbreak in Japan. Shigella is characterized by its strict human host specificity, with very rare instances of infection in other primates. This genus is comprised of four species: *Shigella dysenteriae, Shigella flexneri, Shigella boydii*, and *Shigella sonnei*, each contributing to the disease burden in various geographical regions and with differing levels of severity (Bujňáková *et al*., 2022)

Pathogenicity and Transmission

Shigella is highly adapted to invade the human intestinal mucosa, leading to inflammation and disruption of the colonic epithelium. The bacterium is remarkably infectious, with a low infectious dose of as few as 10 to 100 organisms required to cause disease. Shigellosis primarily manifests as diarrhea, which can range from mild to severe, often containing blood and mucus due to the invasion and destruction of intestinal cells. Fever, abdominal pain, and tenesmus are also common symptoms (Patel *et al*., 2020).

Transmission of Shigella occurs via the fecal-oral route, facilitated by its ability to survive the acidic gastric environment. Outbreaks are often associated with conditions of overcrowding and poor sanitation, making shigellosis a significant concern in developing countries and in settings with inadequate access to clean water and sanitation facilities. Additionally, Shigella can be spread through contaminated food and water, as well as through person-to-person contact (Bujňáková *et al*., 2022).

Antibiotic Resistance in Shigella

The treatment of shigellosis has become increasingly challenging due to the rapid emergence of antibiotic-resistant strains of Shigella. Resistance to traditional first-line antibiotics, such as ampicillin and trimethoprim-sulfamethoxazole, is widespread, and multidrug-resistant (MDR) strains have emerged, displaying resistance to multiple antibiotic classes. Alarmingly, resistance to newer antibiotics, including fluoroquinolones and third-generation cephalosporins, has also been reported (Lee *et al*., 2022).

The mechanisms of resistance in Shigella include chromosomal mutations that confer reduced susceptibility to antibiotics and the acquisition of resistance genes through horizontal gene transfer. The latter is particularly concerning as it allows for the rapid dissemination of resistance traits within and between bacterial populations. The global spread of antibiotic-resistant Shigella strains underscores the urgent need for enhanced surveillance, judicious use of antibiotics, and the development of new therapeutic strategies (Chen *et al*., 2021).

Public Health Implications and Control Measures

Shigellosis represents a significant public health challenge, particularly in resource-limited settings where the burden of disease is highest. Control measures are centered around improving sanitation and hygiene practices to prevent the transmission of the bacterium. This includes ensuring access to clean water, promoting handwashing with soap, and improving food safety practices (Lee *et al*., 2022).

Vaccination represents a promising avenue for the prevention of shigellosis, although no vaccine is currently available for widespread use. Research efforts are focused on developing safe and effective vaccines, which could significantly reduce the incidence of disease, especially among children in endemic regions (Lee *et al*., 2022).

Public health interventions also emphasize the importance of rapid identification and treatment of cases to mitigate outbreaks. However, the growing challenge of antibiotic resistance necessitates a cautious approach to the use of antimicrobial agents, highlighting the need for alternative treatment strategies and the development of novel antibiotics (Patel *et al*., 2020).

4. Klebsiella

Klebsiella pneumoniae: Known for causing a range of infections, including pneumonia, bloodstream infections, wound or surgical site infections, and meningitis, especially in hospital settings. Klebsiella species are particularly concerning due to their high levels of antibiotic resistance (Touati and Majri, 2020).

Klebsiella is a genus of Gram-negative, rod-shaped bacteria belonging to the Enterobacterales order, recognized for its significance in both healthcare settings and the community due to its role in various infections and its capacity for developing antibiotic resistance. Among the species within this genus, *Klebsiella pneumoniae* is the most clinically important, known for causing a wide range of infections including pneumonia, urinary tract infections (UTIs), bloodstream infections, and infections in wounds and surgical sites (Binsker *et al*., 2020).

Pathogenic Features of Klebsiella

Klebsiella species are opportunistic pathogens that can cause disease when the host's defense mechanisms are compromised. They are naturally found in the environment, such as in soil and water, and are also part of the normal flora of the human gastrointestinal tract (Binsker *et al*., 2020). The pathogenicity of Klebsiella, particularly *K. pneumoniae*, is attributed to various virulence factors, including capsular polysaccharides that confer resistance to phagocytosis, fimbriae that facilitate adhesion to host tissues, and siderophores that enable iron acquisition from the host, crucial for bacterial growth and survival (Lee *et al*., 2022).

Klebsiella pneumoniae is notorious for causing hospital-acquired infections, particularly among patients with weakened immune systems, those in intensive care units, or those undergoing invasive procedures (Touati and Majri, 2020). Community-acquired infections by hypervirulent strains of *K. pneumoniae* have also been reported, characterized by severe outcomes such as liver abscesses and meningitis, even in healthy individuals (Touati and Majri, 2020).

Antibiotic Resistance in Klebsiella

A major concern with Klebsiella infections is the high level of antibiotic resistance observed in some strains, rendering standard treatments ineffective. *Klebsiella pneumoniae*, in particular, has been identified as a critical priority pathogen by the World Health Organization (WHO) due to its capacity to acquire resistance mechanisms (Bonnin *et al*., 2021). Multidrug-resistant (MDR) strains, including those producing extended-spectrum beta-lactamases (ESBLs) and carbapenemases, pose significant challenges in clinical management (Touati and Majri, 2020).

Carbapenem-resistant K. pneumoniae (CRKP) strains are especially problematic because carbapenems are often used as a last-resort class of antibiotics for treating severe bacterial infections. Resistance mechanisms involve the production of carbapenemases, enzymes that hydrolyze carbapenems and other beta-lactams, rendering them ineffective (Bonnin *et al*., 2021). The spread of carbapenemase-producing Klebsiella is a global public health threat, associated with high mortality rates due to limited treatment options (Touati and Majri, 2020).

Public Health Implications and Control Strategies

The emergence of antibiotic-resistant Klebsiella strains necessitates stringent infection control measures in healthcare settings to prevent the spread of these pathogens. Strategies include adherence to hand hygiene protocols, the use of personal protective equipment (PPE) (Patel *et al*., 2020), environmental cleaning and disinfection, and the isolation of infected or colonized patients. Surveillance systems are crucial for monitoring the prevalence of resistant strains and informing antibiotic stewardship programs, which aim to optimize the use of antibiotics to reduce the selection pressure that drives resistance (Tompkins van Duiin, 2021).

Research into novel therapeutic approaches, including new antibiotics, bacteriophage therapy, and strategies to inhibit virulence factors or resistance mechanisms, is essential to combat Klebsiella infections effectively. Additionally, understanding the genetic and molecular basis of antibiotic resistance and virulence in Klebsiella can aid in the development of diagnostic tools for rapid detection of resistant strains and tailored therapeutic interventions (Camargo *et al*., 2022).

Enterobacter

Enterobacter, a genus within the Enterobacterales order, consists of Gram-negative, rod-shaped bacteria known for their role in various opportunistic infections. These bacteria are commonly found in the environment, including water, soil, and vegetation, and can also be part of the normal gut flora in humans and animals. Despite their widespread presence, certain species within the Enterobacter genus, such as *Enterobacter cloacae* and *Enterobacter aerogenes*, have gained significance in clinical settings due to their ability to cause healthcare-associated infections (HAIs) and their propensity for developing antibiotic resistance (Tompkins van Duiin, 2021).

Clinical Relevance of Enterobacter

Enterobacter species are capable of causing a broad spectrum of infections, particularly in patients with compromised immune systems or those exposed to hospital environments. These infections can range from urinary tract infections (UTIs) and respiratory tract infections to more severe conditions such as bloodstream infections (sepsis), meningitis, and wound infections. The ability of Enterobacter to inhabit various medical devices, including catheters and ventilators, further contributes to its role in HAIs (Lee *et al.*, 2022).

The virulence of Enterobacter is attributed to several factors, including its capacity to form biofilms on surfaces, which enhances its survival and persistence in hospital settings. Additionally, Enterobacter species possess a variety of virulence factors, such as adhesins that facilitate attachment to host tissues, and endotoxins that trigger inflammatory responses (Tompkins van Duiin, 2021).

Antibiotic Resistance in Enterobacter

A significant challenge in treating Enterobacter infections is the genus's inherent ability to acquire and disseminate antibiotic resistance mechanisms. Enterobacter species have been observed to produce extended-spectrum beta-lactamases (ESBLs) and AmpC beta-lactamases, enzymes that confer resistance to a wide range of beta-lactam antibiotics, including penicillins and cephalosporins. This resistance complicates the selection of effective antimicrobial therapies and often necessitates the use of more potent antibiotics, such as carbapenems (Chen *et al*., 2021).

However, the emergence of carbapenem-resistant Enterobacter species has further exacerbated the challenge of managing infections caused by these bacteria. Resistance to carbapenems in Enterobacter can be mediated through the production of carbapenemase enzymes, porin mutations that reduce antibiotic uptake, or efflux pumps that actively expel antibiotics from the bacterial cell. The presence of carbapenem-resistant Enterobacter in healthcare settings is particularly concerning due to the limited treatment options available and the high risk of transmission to vulnerable patient populations (Touati and Majri, 2020).

Public Health Implications and Control Strategies

The increasing prevalence of antibiotic-resistant Enterobacter strains highlights the need for comprehensive infection control measures in healthcare facilities. Strategies to prevent the spread of Enterobacter include strict adherence to hand hygiene practices, the use of personal protective equipment (PPE) by healthcare workers, environmental cleaning and disinfection, and the implementation of antibiotic stewardship programs to optimize antibiotic use and reduce selective pressure for resistance (Binsker *et al*., 2020).

Surveillance and rapid diagnostic testing are crucial for the early detection of antibiotic-resistant Enterobacter strains, allowing for timely intervention and the appropriate management of infected patients. Research into novel therapeutic options, including new antimicrobial agents, bacteriophage therapy, and strategies targeting bacterial virulence mechanisms, is essential to address the growing challenge posed by Enterobacter and other multidrug-resistant organisms (Binsker *et al*., 2020).

5. Yersinia

Yersinia is a genus of bacteria within the order Enterobacterales that includes species known for their significant impact on human health. Among the 11 species identified within this genus, three are particularly notable for causing human diseases: Yersinia pestis, *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis* (Patel *et al*., 2020). Each of these species is associated with distinct clinical manifestations and epidemiological patterns, highlighting the diverse pathogenic potential within the Yersinia genus (Touati and Majri, 2020).

Yersinia pestis and Plague

Yersinia pestis is the etiological agent of plague, one of the most devastating infectious diseases in human history, responsible for several pandemics including the Black Death in the 14th century. Plague presents in three forms: bubonic, pneumonic, and septicemic. Bubonic plague, the most common form, is transmitted to humans through the bite of infected fleas that have fed on infected rodents. Pneumonic plague, the most virulent form, can be spread through aerosol droplets from person to person. Septicemic plague occurs when the infection spreads directly to the bloodstream (Binsker *et al*., 2020).

Despite being perceived as a historical disease, plague still persists in natural foci around the world, with occasional outbreaks reminding us of its potential for human impact. Modern antibiotics are effective against *Y. pestis*, but prompt treatment is crucial to prevent serious outcomes or death (Camargo *et al*., 2022).

Enteric Infections

Yersinia enterocolitica and *Yersinia pseudotuberculosis* are primarily associated with enteric infections, presenting a spectrum of symptoms ranging from mild gastroenteritis to more severe conditions such as terminal ileitis and mesenteric lymphadenitis, which can mimic appendicitis (Camargo *et al*., 2022). These species are zoonotic pathogens transmitted to humans primarily through the consumption of contaminated food products, particularly pork (for Y. enterocolitica) and vegetables contaminated by animal feaces (Camargo *et al*., 2022).

Y. enterocolitica and *Y. pseudotuberculosis* infections are more common in cooler climates, and although they generally result in self-limiting diseases, complications can occur, especially in immunocompromised individuals. The ability of these bacteria to survive and multiply at refrigeration temperatures contributes to their transmission through contaminated foods (Lee *et al*., 2022).

Antibiotic Resistance and Public Health Challenges

The issue of antibiotic resistance is less pronounced in Yersinia compared to other Enterobacterales, but resistance patterns do exist and can complicate treatment options, particularly in resource-limited settings. Monitoring resistance patterns and maintaining effective antibiotic stewardship programs are essential to managing infections caused by these pathogens (Camargo *et al*., 2022).

Control and Prevention

Controlling Yersinia infections involves a multifaceted approach that includes surveillance of human and animal populations, vector control measures for plague, and ensuring food safety to prevent enteric Yersinia infections. Public health efforts also focus on educating at-risk populations about prevention strategies and the importance of early treatment seeking for suspected cases (Tompkins van Duiin, 2021).

For Y. pestis, efforts to control rodent populations and reduce the risk of flea bites in endemic areas are crucial. For *Y. enterocolitica* and *Y. pseudotuberculosis*, measures include promoting good hygiene practices in food handling and preparation to prevent cross-contamination and the consumption of undercooked meat (Bujňáková *et al*., 2022).

6. Proteus

Proteus is a genus of Gram-negative bacteria that belongs to the Enterobacterales order. Known for their distinctive ability to exhibit swarming motility on solid surfaces, Proteus species are widely distributed in the environment, including soil, water, and the intestinal tracts of humans and animals. The most clinically significant species within this genus are Proteus mirabilis and, to a lesser extent, Proteus vulgaris, both of which are implicated in human infections (Camargo *et al*., 2022).

Clinical Significance

Proteus species, particularly Proteus mirabilis, are notable pathogens in the clinical setting, primarily associated with urinary tract infections (UTIs). They are capable of causing both community-acquired and hospital-acquired infections. Proteus bacteria can infect the bladder (cystitis), kidneys (pyelonephritis), or may form stones (urolithiasis) within the urinary tract. The ability of these bacteria to produce urease, an enzyme that hydrolyzes urea to ammonia and carbon dioxide, increases urine pH and contributes to the formation of kidney stones. Additionally, Proteus infections may lead to complications such as bacteremia, especially in patients with underlying health conditions or those with indwelling urinary devices (Camargo *et al.*, 2022).

Pathogenesis and Antibiotic Resistance

The pathogenesis of Proteus infections involves colonization and invasion facilitated by virulence factors such as fimbriae (adherence factors), flagella (motility), and the aforementioned urease enzyme. The swarming motility of Proteus, characterized by rapid and coordinated movement of bacterial cells across surfaces, aids in colonization and the establishment of infections (Rabaan *et al.*, 2022).

Antibiotic resistance in Proteus species, especially P. mirabilis, is a growing concern. These bacteria have demonstrated resistance to commonly used antibiotics, including ampicillin, tetracycline, and sulfonamides. More worryingly, increased resistance to more potent antibiotics such as fluoroquinolones and third-generation cephalosporins has been observed (Rahbe *et al.*, 2024). Resistance mechanisms include the production of beta-lactamases, efflux pumps, and alterations in antibiotic targets. The presence of extended-spectrum beta-lactamases (ESBLs) and carbapenemases in some Proteus strains poses significant challenges to treatment, necessitating the use of carbapenems or other last-resort antibiotics for some infections (Rodríguez-Santiago *et al.*, 2021).

Public Health and Clinical Management

The management of infections caused by Proteus species requires accurate identification of the pathogen and susceptibility testing to guide antibiotic therapy. In the case of UTIs, removing or managing any predisposing factors, such as urinary catheters, is also crucial.

Preventive measures include strict adherence to infection control practices in healthcare settings to prevent the spread of resistant strains (Livermore *et al*., 2020).

Addressing antibiotic resistance in Proteus and other bacterial pathogens necessitates a multifaceted approach. This includes promoting antibiotic stewardship to ensure the judicious use of antibiotics, enhancing infection prevention and control measures in healthcare and community settings, and investing in research to understand resistance mechanisms and develop new therapeutic options (Bujňáková *et al*., 2022).

7. Citrobacter

Citrobacter is a genus of Gram-negative bacteria that belongs to the family Enterobacteriaceae, within the order Enterobacterales. These bacteria are widely distributed in the environment, found in water, soil, and the intestinal tracts of animals and humans. They are facultatively anaerobic, rod-shaped, and capable of performing both fermentative and respiratory metabolism. Citrobacter species, while often commensal organisms, can also act as opportunistic pathogens, causing a range of infections in humans (Bujňáková *et al*., 2022).

Clinical Significance

The most commonly encountered species in clinical settings are *Citrobacter freundii* and *Citrobacter koseri*. Citrobacter species can cause a variety of infections, including urinary tract infections (UTIs), respiratory tract infections, bacteremia, wound infections, and, less commonly, meningitis and brain abscesses, particularly in neonates and immunocompromised individuals (Livermore *et al*., 2020). *Citrobacter koseri*, in particular, is known for its association with neonatal meningitis and the formation of brain abscesses, which can lead to significant morbidity and mortality (Camargo *et al*., 2022).

Pathogenesis

The pathogenic potential of Citrobacter species is attributed to various virulence factors, including adhesins that facilitate attachment to host tissues, siderophores that sequester iron from the host environment, and endotoxins. These bacteria have the ability to colonize and breach the mucosal barriers of the host, leading to systemic infection. In the case of *Citrobacter koseri*, the ability to invade the central nervous system and cause meningitis is particularly concerning (Rabaan *et al*., 2022).

Antibiotic Resistance

Similar to many Enterobacterales, *Citrobacter species* have shown an increasing trend in antibiotic resistance, complicating the treatment of infections. They are capable of producing extended-spectrum beta-lactamases (ESBLs) and AmpC beta-lactamases, enzymes that confer resistance to penicillins, cephalosporins, and, in some cases, carbapenems (Rabaan *et al*., 2022). Resistance to other classes of antibiotics, such as fluoroquinolones and aminoglycosides, has also been reported. The presence of mobile genetic elements, such as plasmids and transposons, facilitates the horizontal transfer of resistance genes among Citrobacter and other bacteria, further exacerbating the challenge of antibiotic resistance (Livermore *et al*., 2020).

Clinical Management and Public Health Implications

The management of infections caused by Citrobacter species requires accurate microbiological diagnosis and susceptibility testing to guide appropriate antibiotic therapy. In cases of serious infections, such as meningitis or bacteremia, combination antibiotic therapy may be necessary to achieve effective treatment (Livermore *et al*., 2020). The emergence of antibiotic-resistant Citrobacter strains underscores the need for vigilant antibiotic stewardship practices in both healthcare and community settings to curb the spread of resistance.

Preventive measures, including rigorous infection control practices in healthcare facilities and the promotion of good hygiene practices, are essential to minimize the transmission of Citrobacter and other opportunistic pathogens (Rabaan *et al*., 2022). Continued surveillance for antibiotic-resistant strains and research into the mechanisms of resistance and pathogenesis of Citrobacter infections are crucial for developing effective strategies to treat and prevent these infections (Rodríguez-Santiago *et al*., 2021).

2.5.1 Enterobacterales In Composted Manure

The Enterobacterales order, encompassing a diverse array of Gram-negative bacteria, plays a significant role in various environments, including composted manure, where they contribute to nutrient cycling and the decomposition process. Composted manure, an organic material resulting from the aerobic decomposition of animal feces, is widely used in agriculture to improve soil fertility and structure (Katada *et al*., 2021). The microbial community within composted manure is complex and dynamic, with Enterobacterales members being pivotal due to their metabolic versatility and ability to break down complex organic materials (Esperón *et al*., 2020).

Role of Enterobacterales in Composted Manure

Enterobacterales bacteria are facultatively anaerobic, thriving in both oxygen-rich and oxygen-poor conditions, which makes them particularly suited to the fluctuating conditions within compost piles. These bacteria participate in the degradation of organic matter, converting it into simpler compounds that are more easily utilized by plants and other organisms in the soil. This decomposition process not only recycles nutrients but also reduces the volume of waste, contributing to environmental sustainability (Beauchemin *et al*., 2022).

During the composting process, the activity of Enterobacterales and other microorganisms generates heat, which can significantly raise the temperature of the compost pile. This thermophilic phase is crucial for the effective decomposition of organic material, as well as for the sanitation of the compost by killing pathogens and weed seeds. Enterobacterales bacteria, due to their robustness, can survive and remain active in varying temperatures, playing a role throughout the composting process (Keenum *et al*., 2021).

While the presence of Enterobacterales in composted manure is beneficial for decomposition and nutrient cycling, it also raises public health concerns. Some members of the Enterobacterales order, such as certain strains of Escherichia coli and Salmonella, are pathogenic to humans and animals (Anderson *et al*., 2023). The survival of these pathogens in composted manure and their potential transmission through the food chain, particularly via the application of manure to edible crops, is a matter of concern (Zhan *et al*., 2020).

To mitigate these risks, proper composting practices are essential. Adequate aeration, moisture control, and achieving and maintaining high temperatures for a sufficient period during the composting process are key factors in reducing the levels of pathogens to safe levels. Additionally, regulations and guidelines on the application of composted manure to agricultural land aim to prevent contamination of food crops and protect public health (Katada *et al*., 2021).

The Enterobacterales, a diverse order of Gram-negative bacteria, play a pivotal role in nutrient cycling, a fundamental ecological process that recycles elements and compounds in various forms and makes them available to living organisms (Esperón *et al*., 2020). This group includes a variety of genera, such as Escherichia, Salmonella, Klebsiella, and Enterobacter, which are involved in the decomposition of organic matter and the transformation of nutrients in soil, water, and within host organisms. Their activities are crucial in ecosystems, influencing soil fertility, plant growth, and the health of aquatic environments (Beauchemin *et al.*, 2022).

Nitrogen Cycling

One of the most critical elements in nutrient cycling is nitrogen, essential for the synthesis of proteins, nucleic acids, and other cellular constituents. Enterobacterales contribute significantly to the nitrogen cycle through processes such as nitrogen fixation, nitrification, denitrification, and ammonification (Rabaan *et al.*, 2022).

- **Nitrogen Fixation:** Certain Enterobacterales, particularly those forming symbiotic relationships with plants, can convert atmospheric nitrogen into ammonia, a form that plants can readily assimilate. This process is crucial in natural ecosystems and agricultural settings, where it reduces the need for synthetic nitrogen fertilizers (Keenum *et al.*, 2021).
- **Ammonification:** Enterobacterales decompose organic nitrogenous materials (e.g., dead cells, waste products) into ammonia or ammonium ions through ammonification. This process is vital in recycling nitrogen within ecosystems, making it available for use by plants and other microorganisms.
- **Denitrification:** Some members of Enterobacterales can perform denitrification, converting nitrates in the soil back into nitrogen gas, which is then released into the atmosphere. This process is crucial in regulating the global nitrogen cycle but can also contribute to the loss of soil fertility in agricultural lands (Zhan *et al.*, 2020).

Phosphorus and Sulfur Cycling

Beyond nitrogen, Enterobacterales are involved in the cycling of other essential nutrients, including phosphorus and sulfur, both of which are vital for living organisms.

- **Phosphorus:** Enterobacterales participate in the breakdown of organic phosphorus compounds, releasing inorganic phosphate into the soil, where it can be absorbed by plants. Phosphorus is a critical component of nucleic acids, ATP, and phospholipids, and its availability is often a limiting factor in ecosystems (Beauchemin *et al*., 2022).
- **Sulfur:** These bacteria also play a role in sulfur cycling, breaking down organic sulfur compounds to produce hydrogen sulfide (H_2S) or transforming inorganic sulfates into organic sulfur forms. Sulfur is essential for the synthesis of amino acids, vitamins, and other biomolecules (Zhan *et al*., 2020).

Impact on Agriculture and Environmental Sustainability

The activities of Enterobacterales in nutrient cycling have direct implications for agriculture and environmental sustainability. By converting nutrients into forms that are usable by plants, these bacteria enhance soil fertility and promote plant growth, contributing to the productivity of natural and agricultural ecosystems. The efficient cycling of nutrients also supports the resilience of ecosystems against disturbances and reduces the reliance on chemical fertilizers, which can have detrimental environmental impacts (Keenum *et al*., 2021).

2.5.2 Public Health Implications

The presence of colistin-resistant Enterobacteriales in composted manure poses significant public health implications, highlighting a complex interface between agricultural practices, environmental health, and human health. Colistin, a last-resort antibiotic for treating multidrug-resistant Gram-negative bacterial infections, has become increasingly important in the face of rising antibiotic resistance (Zhan *et al.*, 2020). The discovery of colistin-resistant Enterobacterales, particularly those carrying the plasmid-mediated mcr-1 gene and its variants, in environments such as composted manure, underscores the potential for antibiotic resistance genes (ARGs) to disseminate through agricultural and natural ecosystems (Zhan *et al.*, 2020).

Transmission Pathways to Humans

The use of composted manure as a soil amendment in agriculture is a common practice due to its beneficial effects on soil fertility and structure. However, if this manure contains colistin-resistant Enterobacteriales, it can become a conduit for the spread of resistance genes to other bacteria in the soil, crops, and, ultimately, to humans and animals. Transmission pathways include:

Consumption of Contaminated Produce: Vegetables and fruits grown in soil amended with contaminated compost can harbour resistant bacteria, posing a risk to consumers who ingest these products.

Environmental Dissemination: Runoff from agricultural lands can carry resistant bacteria and ARGs to water bodies, affecting aquatic ecosystems and potentially contaminating drinking water sources (Zhan *et al.*, 2020).

Direct Contact: Farmers and agricultural workers handling composted manure or working in fields treated with such manure may be directly exposed to resistant bacteria (Tompkins van Duiin, 2021).

Public Health Risks

The spread of colistin resistance poses several public health risks, including:

Increased Burden of Infections: The presence of colistin-resistant bacteria in the environment can lead to an increased incidence of infections that are difficult to treat, requiring the use of alternative, potentially less effective, or more toxic antibiotics.

Limited Treatment Options: The efficacy of colistin as a last-resort treatment is compromised by the spread of resistance, leaving few therapeutic options for patients with infections caused by multidrug-resistant bacteria (Touati and Majri, 2020).

Healthcare Costs: Infections caused by resistant bacteria often result in prolonged hospital stays, increased healthcare costs, and a higher risk of mortality.

2.5.3 Factors Affecting The Prevalence Of Colistin Antibiotic Resistance

The prevalence of antibiotic resistance among bacteria, including those in the Enterobacterales order, is a consequence of various factors that contribute to the selection, propagation, and spread of resistant strains (Claudia *et al*., 2023). This complex issue is driven by both natural microbial evolution and human activities, making it one of the most daunting challenges in public health today. The following factors are key contributors to the increasing prevalence of antibiotic resistance:

1. Overuse and Misuse of Antibiotics

One of the primary drivers of antibiotic resistance, including resistance to colistin, is the overuse and misuse of antibiotics in both human medicine and veterinary practices. Inappropriate prescribing by healthcare providers, such as prescribing antibiotics for viral infections against which they are ineffective, contributes significantly to the problem (Kawamoto *et al*., 2022). Additionally, patients not completing their prescribed antibiotic courses or using antibiotics without proper medical guidance can allow partially resistant bacteria to survive and proliferate. This indiscriminate use of antibiotics exerts selective pressure on bacterial populations, encouraging the emergence and spread of resistance mechanisms (Alkofide *et al*., 2020).

2. Use of Antibiotics in Agriculture

The agricultural sector is a major consumer of antibiotics worldwide, using them not only for treating sick animals but also for growth promotion and disease prevention in livestock and aquaculture. This extensive use of antibiotics, including colistin, in animal husbandry has been linked to the emergence of resistant bacterial strains. Resistant bacteria can be transmitted to humans through direct contact with animals, the consumption of contaminated meat and other animal products, and environmental pathways, such as runoff from farms contaminating water sources (Alkofide *et al*., 2020). The widespread presence of antibiotics in the environment also selects for resistance genes that can be transferred to human pathogens, further complicating the fight against antibiotic resistance.

3. Hospital and Healthcare Settings

Hospitals and other healthcare settings are hotspots for the emergence and transmission of antibiotic-resistant bacteria, including colistin-resistant strains. The high use of antibiotics in these settings, particularly for patients with compromised immune systems or those undergoing invasive procedures, creates an environment conducive to the development of resistance (Kawamoto *et al*., 2022). Inadequate infection control practices can facilitate the spread of resistant bacteria among patients, healthcare workers, and the community at large. The emergence of colistin resistance in healthcare settings is particularly concerning given its status as a last-resort antibiotic for treating infections caused by multidrug-resistant organisms (Kawamoto *et al*., 2022).

4. Environmental Factors

The environment plays a significant role in the spread of antibiotic resistance. Antibiotics from human and veterinary medicine, as well as from agricultural use, can enter the environment through various routes, including wastewater, manure application, and pharmaceutical manufacturing waste. These antibiotics can select for resistant bacteria in soil and water, which can then be transferred to animals and humans (Alkofide *et al*., 2020). The presence of antibiotic resistance genes (ARGs) in environmental bacteria is a reservoir for resistance that can potentially be transferred to human pathogens. Moreover, changes in the environment, such as pollution and climate change, can impact the distribution and dynamics of bacterial populations, influencing the spread of antibiotic resistance (Claudia *et al*., 2023).

5. Socioeconomic Factors

Socioeconomic factors play a crucial role in the prevalence and spread of antibiotic resistance, including resistance to colistin. These factors encompass a wide range of issues, including access to healthcare, education, and sanitation, which can significantly impact the dynamics of antibiotic use and the spread of resistant bacteria.

Limited access to healthcare in low- and middle-income countries often leads to the inappropriate use of antibiotics, including self-medication and the use of non-prescribed antibiotics. This practice is partly due to the lack of diagnostic facilities that can confirm bacterial infections and recommend specific treatments, leading to the misuse of broad-spectrum antibiotics like colistin, which accelerates the development of resistance (Alkofide *et al*., 2020).

Lack of public awareness and education about antibiotics and antibiotic resistance contributes to their misuse. Without adequate knowledge, individuals may not follow prescribed antibiotic courses, use antibiotics intended for others, or seek antibiotics for viral infections, all of which can promote the emergence and spread of resistance. Poor sanitation and hygiene practices in both healthcare settings and the community can facilitate the transmission of resistant bacteria (Claudia *et al.*, 2023). In areas lacking proper waste disposal and clean water supply, the risk of infection increases, leading to a higher reliance on antibiotics and, consequently, the development of resistance. Economic disparities can exacerbate the spread of antibiotic resistance. In wealthier countries, the high consumption of antibiotics in both humans and animals can lead to the emergence of resistance, which can spread globally through travel and trade. Conversely, in poorer regions, the lack of access to essential medicines can lead to untreated infections and the use of substandard or counterfeit antibiotics, further driving resistance (Kawamoto *et al.*, 2022).

6. Lack of New Antibiotics

The slow pace of new antibiotic development is a significant factor contributing to the growing challenge of antibiotic resistance, including resistance to colistin. Several factors contribute to this issue (Kawamoto *et al.*, 2022). Developing new antibiotics is a costly and time-consuming process with a relatively low financial return on investment compared to other types of pharmaceuticals. This economic reality has led to a decrease in research and development efforts within the pharmaceutical industry focused on discovering new antibiotics (Claudia *et al.*, 2023).

Stringent regulatory requirements for the approval of new antibiotics can also deter pharmaceutical companies from investing in antibiotic research. Demonstrating the safety and efficacy of new antibiotics requires extensive clinical trials, further increasing development costs (Alkofide *et al.*, 2020). The discovery of novel antibiotics that are effective against multidrug-resistant bacteria is scientifically challenging. Many of the "easy targets" for antibiotic action have already been exploited, and finding new mechanisms of action that can bypass existing resistance mechanisms requires innovative approaches and substantial research efforts (Zhan *et al.*, 2020).

The lack of new antibiotics entering the market means that existing antibiotics, including colistin, are used more extensively, increasing the pressure on these drugs and accelerating the development of resistance. Addressing this gap requires incentives for antibiotic research and development, including public-private partnerships, streamlined regulatory processes, and innovative funding models that can encourage the pharmaceutical industry to invest in the discovery of new antimicrobial agents (Kawamoto *et al.*, 2022).

7. Genetic Factors of Bacteria

The genetic factors of bacteria play a critical role in the development and dissemination of antibiotic resistance, including resistance to colistin. Bacteria have evolved a variety of genetic mechanisms that enable them to resist the effects of antibiotics, facilitating their survival in environments where antibiotics are present. Understanding these genetic factors is crucial for developing strategies to combat antibiotic resistance (Touati and Majri, 2020).

Mutation

One of the fundamental genetic mechanisms that contribute to antibiotic resistance is mutation. Spontaneous mutations in bacterial DNA can alter the structure of antibiotic target sites, reduce drug uptake, or increase drug efflux, rendering antibiotics less effective. For instance, mutations in the genes encoding the bacterial ribosome can lead to resistance to macrolides and aminoglycosides, while mutations in DNA gyrase genes can confer resistance to fluoroquinolones. Mutations can also enhance the bacterium's ability to metabolize or inactivate antibiotics (Camargo *et al.*, 2022).

Horizontal Gene Transfer

Bacteria can acquire antibiotic resistance genes from other bacteria through horizontal gene transfer (HGT), significantly accelerating the spread of resistance. The main mechanisms of HGT include:

- **Conjugation:** This process involves the direct transfer of DNA, typically plasmids carrying resistance genes, from one bacterium to another through cell-to-cell contact. Conjugation can occur between bacteria of the same species or different species, allowing for the widespread dissemination of resistance traits (Tompkins van Duiin, 2021).
- **Transformation:** Bacteria can take up free DNA fragments from their environment, which may include antibiotic resistance genes released by dead bacteria. If these genes are incorporated into the genome, the recipient bacterium can express the resistance trait (Touati and Majri, 2020).
- **Transduction:** Bacteriophages, viruses that infect bacteria, can accidentally package bacterial DNA, including resistance genes, during the viral replication process. When these bacteriophages infect new bacterial cells, they can deliver these genes, leading to the spread of resistance (Touati and Majri, 2020).

Mobile Genetic Elements

Antibiotic resistance genes are often located on mobile genetic elements (MGEs) such as plasmids, transposons, and integrons, which facilitate their movement within and between bacterial genomes. Plasmids can carry multiple resistance genes, conferring resistance to different classes of antibiotics simultaneously, a phenomenon known as multidrug resistance (MDR). Transposons and integrons can capture and mobilize resistance genes, allowing for their integration into various genomic locations and the assembly of complex resistance determinants (Touati and Majri, 2020).

Genetic Factors Specific to Colistin Resistance

Colistin resistance, particularly plasmid-mediated resistance, is primarily associated with the acquisition of mcr genes (mobilized colistin resistance). The mcr-1 gene was the first to be identified and has been found in various bacterial species across different environments and geographic regions. These genes encode enzymes that modify the lipid A component of the bacterial outer membrane, reducing colistin's ability to bind and disrupt the membrane. The spread of mcr genes via plasmids and other MGEs highlights the role of genetic factors in the global dissemination of colistin resistance (Bonnin *et al*., 2021).

2.5.4 Ways to Address Concerns About Transmission To Humans Through Agricultural Practices Or Environmental Exposure

Addressing concerns about the transmission of antibiotic-resistant bacteria, including colistin-resistant Enterobacteriales, to humans through agricultural practices or environmental exposure requires a comprehensive and multifaceted approach (Binsker *et al*., 2020). These strategies involve a combination of improved agricultural practices, regulatory measures, public health initiatives, and scientific research. The goal is to minimize the emergence and spread of antibiotic resistance while ensuring food safety and environmental protection.

Improved Agricultural Practices

It has been suggested that the implementation of antibiotic stewardship programs in veterinary medicine should be a priority, advocating for the judicious use of antibiotics in livestock (Claudia *et al*., 2023). This approach emphasizes the necessity of prescribing antibiotics only when absolutely required, based on precise diagnoses and susceptibility testing, while also recommending against the utilization of antibiotics critical for human health as growth promoters in animals. Furthermore, the development and adoption of best practices for manure management have been proposed to mitigate the presence of antibiotic-resistant bacteria. Techniques such as composting at sufficiently high temperatures to eliminate pathogenic bacteria and ensuring proper storage and treatment before the application of manure are advised to significantly lower risks (Tompkins van Duiin, 2021).

In addition, the strengthening of biosecurity practices on farms has been highlighted as a crucial measure to prevent the introduction and dissemination of infectious agents. This involves controlling access to animal rearing areas, implementing quarantine measures for both new and sick animals, and maintaining the cleanliness of facilities and equipment (Touati and Majri, 2020).

Regulatory and Policy Measures

The enactment and enforcement of regulations restricting the non-therapeutic use of antibiotics in agriculture have been recommended, particularly those antibiotics that are critical for human health. The establishment of surveillance systems to monitor antibiotic use in agriculture and the prevalence of antibiotic-resistant bacteria in livestock, manure, soil, water, and crops has also been proposed. This surveillance is deemed essential for assessing risks and informing policy decisions (Bonnin *et al*., 2021). Moreover, the development and enforcement of guidelines or regulations concerning the application of manure to agricultural lands have been suggested, including recommendations on treatment processes, application rates, and timing to minimize the risk of transmitting resistant bacteria to humans (Claudia *et al*., 2023).

Public Health Initiatives

For public health initiatives, educating farmers, agricultural workers, and the general public about the risks associated with antibiotic resistance and the importance of preventive measures has been emphasized (Claudia *et al.*, 2023). Awareness programs promoting best practices in food handling, personal hygiene, and responsible antibiotic use are recommended. Additionally, fostering collaboration among the agricultural sector, public health authorities, environmental agencies, and the community has been highlighted as a method to tackle the complex challenges posed by antibiotic resistance effectively (Camargo *et al.*, 2022).

Scientific Research and Innovation

Investing in research to discover alternatives to antibiotics for disease prevention and growth promotion in agriculture, such as vaccines, probiotics, prebiotics, and bacteriophage therapy, has been advocated (Touati and Majri, 2020). The exploration and development of technologies and methods for treating environmental contaminants, including antibiotic-resistant bacteria and antibiotic residues in water and soil, are suggested. Advanced filtration, bioremediation, and constructed wetlands are among the techniques believed to hold promise for reducing environmental exposure. Conducting research to better understand the pathways through which antibiotic-resistant bacteria are transmitted from agricultural and environmental sources to humans, and utilizing risk assessment models to identify critical control points and inform targeted interventions, have also been recommended (Claudia *et al.*, 2023).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

This study was carried out in some selected poultry farms within Benin City, Nigeria. Benin City, the study area is in southern Nigeria and is comprised of a population of 3,497,502 people based on the 2006 census (Nigerian Bureau of Statistics, 2011). Benin city is located on Latitude 6°19'3.36"N and Longitude 5°36'52.20"E.

3.2 Study Design

A cross-sectional study which involved collecting data to assess the prevalence of colistin resistant enterobacterales isolated from composted manure in Benin city, Nigeria was carried out..

3.3 Sample Population

Compost manure were collected from About Ten (10) different agricultural sites in Benin City, Nigeria.

3.4 Sampling Technique

The convenience sampling technique was used to sample sites in this study.

3.5 Selection Criteria

Inclusion Criteria

Sites where composted manure is regularly utilized as a soil amendment.

Exclusion Criteria

Sites where composted manure is not used for agricultural purposes.

3.6 Sample Size Determination

The minimum sample size for this study was determined using the World Health Organization standard sample formula for calculation of sample size (Daniel *et al.*, 1999).

N =

Where:

N= desired sample size when population is greater than 10,000

Z = standard normal deviation, usually set at 1.96 (for 95% confidence interval)

P = proportion in the target population estimated to have a particular characteristic.

q= 1.0-p

d= degree of accuracy desired, usually set at 0.05.

From the global pooled prevalence of colistin resistance; (P) of 17.0% = 17.0100 = 0.17 (Ngbede *et al*., 2020).

N =

N = 217

Minimum sample size calculated is 217. In order to account for non-response of participants, 10% of the sample size (i.e., 21.7) will be added to the calculated sample size; this gives a sample size of 238.7, which is approximated to 239 participants.

3.7 Study Population

The sample population were randomly selected diverse agricultural sites in Benin City, Nigeria, where composted manure is routinely used.

3.8 Ethical Approval

Ethical approval for this research was obtained from the Ethics and research committee Ministry of Health, Edo State, Nigeria through their letter referenced HA/737/24/D/04003217 informed consent was also obtained from the site/farm owners. Before collection of the compost manure to indicate their voluntary participation in the study.

3.9 Sample Collection

- **Site Selection:** Sites were identified and randomly selected across Benin City. Sites which represent a mix of livestock and crop farming practices will be used.

- **Composted Manure Sampling:** Composted manure samples was collected from each selected site using sterile containers and tools to avoid cross-contamination. It will be transported immediately to the Medical Microbiology Laboratory, University of Benin Teaching Hospital (UBTH), Edo State for analysis. Socio demographic data accompanying the specimens, such as the particular site and type of livestock reared was obtained from site owners.
- **Sample Preservation:** Proper labelling and immediate preservation of samples was done prior to analysis.

3.10 Sample Analysis

Samples were homogenized in buffered peptone water within 24 hours post collection from the site or farm. The composted manure were cultured on Mac-conkey agar plate and incubated at 37c for 18-24 hours. Isolates were identified based on colonial morphology, motility, lactose fermentation, Gram staining reaction and biochemical tests (indole, citrate, oxidase and urease tests) isolates were subcultured into nutrient agar slant and stored at room temperature until needed.

3.11 Antimicrobial Susceptibility Testing

The antimicrobial susceptibility testing was determined using kirby-Bauer disc diffusion method as per the clinical Laboratory standards institute, (CLSL,2020). With a specific focus on colistin. On Mueller Hinton agar, test organism was emulsified in sterile water and the turbidity matched with 0.5 McFarland standard. once matched, the entire surface of Mueller-Hinton agar plate was flooded with the suspension and excess liquid discarded aseptically. The following antibacterial disc were used: Amoxicillin Clavulanate (30ug), Cefotaxime (25ug), Cefexime (5ug), Imipenem (10ug), Cefuroxime (30ug), Gentamycin (10ug), Nalidixic acid (30ug), Ampiclox (10ug), Levofloxacin (5ug), Ofloxacin (5ug), Nitrofurantoin (300ug), Ceftriaxone sulfbactam (45ug), Colistin (64 ug). The discs were placed equidistance from each other. The plates were incubated at 37°C for 18-24 hours and the zones of inhibition measured in millimeters (mm). The results obtained were interpreted as Sensitive and Resistance in accordance with the Clinical and Laboratory Standards Institute (CLSI, 2020).

- **Minimum Inhibitory Concentration (MIC) SUSCEPTIBILITY TESTING OF COLISTIN:** Minimum inhibitory concentration (MIC) of colistin to the bacteria was carried out for confirmed resistant strains to quantify the level of colistin resistance.Using calcium enhanced Mueller-Hilton broth containing 200µgram/L of calcium. Colistin (colistin sulphate-pantex Holland) was diluted in calcium- enhanced Muller-Hinton broth to cover the range 1.0 – 16 µgram/L. Colonies of the test isolates from an overnight culture was emulsified in sterile water and matched with 0.5 Mcfarland standards. Once matched, the organism suspended contains 10^8cfu/ml. This suspension was diluted 1 in 100 with sterile, water to give 10^6cfu/ml. One mililitre of the 10^6 cfu/ml was added to the various concentration of colistin in the calcium-enhanced Muller- Hinton broth. This resulted in a 1 in 2 dilution of the organism and colistin, thus the final inoculum size was 5×10^5 cfu/ml and colistin concentration ranging from 1.0 to 16 µgram/L. The tubes were incubated at 37°C for 18-24 hours. The MIC was read visually as the least concentration without growth (turbidity). The CLSI MIC breaking point was used for interpretation of the MIC results. Isolates with MIC of $\leq$ 2µg/ml were categorized as susceptible and those with>2µg/ml were categorized as resistant.

3.12 STATISTICAL ANALYSIS

The results obtained from laboratory investigations were tabulated, encoded and statistically analysed using Statistical Package for Social Sciences (SPSS) version 21 program. Chi-square test will be used for analysis (levels of significance will be accepted at $p<0.05$).

CHAPTER FOUR

RESULTS

Table 4.1 shows the general distribution of bacteria isolate samples and the number of samples collected from different farm locations in Benin City. Majority of the isolates recovered were Gram positive bacilli (79/272), followed by Gram positive cocci (72/272). Gram negative bacilli (enterobacterales) had the least recovery rate (4.0%).

Table 4.1 Distribution of Bacteria Isolates from Compost Sites

Bacteria isolate	Number of samples	Percentage (%)
Gram positive cocci	72	26.5
Gram negative bacilli	11	4.0
Gram positive bacilli	79	29.0
Mixed growth of Gram positive bacilli and Gram positive cocci	28	10.3
Mixed growth of Gram positive cocci and aerobic spore bacteria	48	14.7
No growth	34	12.5
Total	272	100

Table 4.2 shows the overall Prevalence of Isolated Enteriobacterales. This data shows the distribution and frequency of different Enterobacterales organisms isolated from compost manure. E. coli was isolated 5 times, comprising 45.5% of the total isolates. *Klebsiella spp*. were isolated 3 times, representing 27.3% of the total. *Proteus spp*. accounted for 2 isolates, making up 18.2% of the total. *Providencia spp*. was isolated once, which is 0.91% of the total. In total, there were 11 Enterobacterales isolates, from of the samples analyzed.

Table 4.2 The Total Prevalence of Isolated Enteriobacterales.

Organism	**No of isolates**	**Percentage**
E. coli	5	45.5
Klebsiella spp	3	27.3
Proteus spp	2	18.2
Providencia spp	1	0.91
Total	11	100

Table 4.3 shows the prevalence distribution of Enterobacterales isolates resistance to colistin. Of the 5 *E. coli* samples isolated, 1(20%) showed colistin resistance. Of the 3 *Klebsiella spp isolated* none showed resistance to colistin. 2 Proteus samples were isolated, all of which showed resistance to colistin. Only one isolate of Providencia was found which showed resistance to colistin in the single isolate found.

Table 4.3 Prevalence of Colistin Resistance of Enterobacterales Recovered From Compost

Organism	**No of isolates**	**Colistin**	
		Susceptible (%) (≤2µg/ml)	**Resistance (%) (>2µg/ml)**
E. coli	5	4(80%)	1(20%)
Klebsiella spp	3	3(100%)	0(0%)
Proteus spp	2	0(0%)	2(100%)
Providencia spp	1	0(0%)	1(100%)
Total	11	7(63.6%)	4(36.4%)

Table 4.4 displays the locations of compost manure sample source. Ugbowo, Isihor, Ekenhuan, Upper Sakponba, Oluku bypass, Ekosodin, Ugbor, Uselu. The highest isolates was gotten from Ugbor area (3) while no isolates was gotten from Isihor and Oluku (0). Two isolates each was gotten from Ugbowo, Ekenhuan and Upper. While Ekosodin and Uselu samples grew one Enterobacterales each.

Table 4.4: Distribution of Enterobacterale isolates from Different Compost Locations

Farm location	**N of isolates**	***E.coli* (%)**	**Kleb (%)**	**Proteus (%)**	***Providencia* (%)**	**X^2**	**p-value**
Ugbowo	2	1(20%)	1(50%)	0(0%)	0(0%)		
Isihor	0	0(20%)	0(0%)	0(0%)	0(0%)		
Ekenhuan	2	1(20%)	0(0%)	0(0%)	1(100%)		
Upper Sakponba	2	0(0.0%)	1(50%)	1(50%)	0(0%)	97.2	0.000
Oluku bypass	0	0(0.0%)	0(0%)	0(0%)	0(0%)		
Ekosodin	1	1(20%)	0(0%)	0(0%)	0(0%)		
Ugbor	3	1(20%)	1(50%)	1(50%)	0(0%)		
Uselu	1	1(20%)	0(0%)	0(50%)	0(0%)		
Total	11	5	3	2	1		

Table 4.5 presents the results from the Minimum Inhibitory Concentration (MIC) testing across the different Enterobacterales species. Of all the enterobacterale isolate recovered, majoriy(7) were colistin sensitive. Four isolates of the genus Proteus, Providencial and Escherichia showed colistin resistance (>2μ/ml).

Table 4.5: Minimum Inhibitory Concentration Values Of Enterobacteriales Recovered From Compost To Colistin Using Broth Microdilution Methods.

Organisms	Minimum Inhibitory Concentration(MIC) (µg/mL)
E. coli	<1
E. coli	<1
E. coli	2
E. coli	2
E. coli	8
Klebsiella spp	<1
Klebsiella spp	2
Klebsiella spp	2
Proteus spp	4
Proteus spp	4
Providencia spp	8

Keys

Sensitive ≤ 2

Resistance > 2

Table 4.6 presents antibiotic susceptibility testing results for different bacterial species against various antibiotics, with the percentage of strains susceptible (S%) to each antibiotic.

For the five *E. coli* isolates tested, there was a complete lack of sensitivity to Imipenem (IMP) and Augmentin (AUG), indicating resistance to these antibiotics. However, these isolates showed a high level of susceptibility to Ofloxacin (OFX), Ceftriaxone (CRO), and Levofloxacin (LBC), with 100% sensitivity. The sensitivity decreased for Cefotaxime (CTX) and colistin (COL) where only 50% of the isolates were sensitive. E. coli Sensitivity to Gentamycin (GN) was high with 60% of the isolates being susceptible. The lowest sensitivity was observed for Ampiclox (ACX), Cefixime (CXM), and a compound under ZEM, each with only 20% of the isolates showing sensitivity, and 40% for an unspecified antibiotic. *Klebsiella spp.* isolates, on the other hand, demonstrated no sensitivity to a group of antibiotics including Imipenem (IMP), Ampiclox (ACX), Cefixime (CXM), Augmentin (AUG), and a compound under ZEM. A moderate level of sensitivity was observed for Ofloxacin (OFX), and Gentamycin (GN) and Colistin (COL). Notably, these isolates were completely sensitive to Ceftriaxone (CRO) and Levofloxacin (LBC), while only 33.3% were sensitive to Cefotaxime (CTX). The two *Proteus spp.* isolates tested showed no susceptibility to the same group of antibiotics as the *Klebsiella spp.* isolates, displaying 0% sensitivity. However, they were entirely susceptible to Ofloxacin (OFX) and Ceftriaxone (CRO). Half of these isolates were sensitive to Cefotaxime (CTX), Levofloxacin (LBC), Gentamycin (GN) and Colistin (COL), Lastly, the single *Providencia spp.* isolate showed no sensitivity to Cefotaxime (CTX), Ampiclox (ACX), Cefixime (CXM), Augmentin (AUG), and Gentamycin (GN) and Colistin (COL), indicating resistance to these antibiotics. Remarkably, this isolate was completely sensitive to the remaining antibiotics tested, including Imipenem

(IMP), Ofloxacin (OFX), Ceftriaxone (CRO), Levofloxacin (LBC), a compound under ZEM, and Colistin (COL).

Figure 4.6: Antimicrobial susceptibility pattern of Enterobacterales isolated

		IMP	OFX	CTX	ACX	CXM	CRO	LBC	ZEM	AUG	GN	COL
		(10)	(30)	(25)	(10)	(30)	(45)	(5)	(5)	(30)	(10)	(64)
	N	S(%)	S(%)	S(%)	S(%)	S(%)	S(%)	S(%)	S(%)	S(%)	S(%)	S(%)
E. coli	5	0(0%)	5(100%)	5(50%)	1(20%)	1(20%)	5(100%)	5(100%)	2(40%)	0(0%)	3(60%)	4(60%)
Klebsiella spp.	3	0(0%)	2(66.7%)	1(33.3%)	0(0%)	0(0%)	3(100%)	3(100%)	0(0%)	0(0%)	2(66.7%)	3(66.7%)
Proteus spp.	2	0(0%)	2(100%)	1(50%)	0(0%)	0(0%)	2(100%)	1(50%)	0(0%)	0(0%)	1(50%)	1(50%)
Providencia spp.	1	0(0%)	1(100%)	0(0%)	0(0%)	0(0%)	1(100%)	1(100%)	1(100%)	0(0%)	0(0%)	1(100%)

Key: S=sensitive, N=number of isolate, IMP=Imipenem, OFX=Ofloxacin, CTX=Cefotaxime, ACS=Ampiclox, CXM=Cefixime, CRO=Ceftriaxone subactem, LBC=Levofloxacin, ZEM=Cefixime, AUG=Augmentin, GN=Gentamycin, COL=Colistin

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1 Discussion

Antimicrobial resistance (AMR) has emerged as a significant global public health threat, compromising the effectiveness of antibiotics, which are cornerstone treatments for bacterial infections (Gogry *et al*., 2021). Enterobacterales, a large family of bacteria that includes well-known pathogens, are particularly concerning due to their high potential to acquire and disseminate resistance genes. Colistin, a "last-resort" antibiotic for multidrug-resistant Gram-negative bacterial infections, has seen increasing use in recent time, leading to the emergence of colistin-resistant strains (Gogry *et al*., 2021). This study therefore sought to investigate the prevalence of colistin resistance among enterobacteriales recovered from compost made from faeces of pigs and poultry,frequently eaten in southern Nigeria.

The presence of Gram-negative bacilli is of particular concern due to the potential for these species to carry and spread genes responsible for antibiotic resistance, such as those conferring resistance to colistin. The findings of several co-habiting bacteria species in compost manure is corroborated by findings of Wang *et al*., (2019). The observation of mixed growths (e.g., Gram-positive bacilli and cocci, Gram-positive cocci and aerobic spore-forming bacteria) suggests a complex microbial community in these environments, which can be a hotspot for horizontal gene transfer, a key mechanism in the spread of antibiotic resistance as discussed by Zhou *et al*., (2021).

The total prevalence of colistin resistance in isolated enterobacteriale is 36.4%(4/11). This is high but comparable with a prevalence of 41% documented by Qadi *et al*., (2021) in a study carried out in the Gaza strip in Asia but lower than colistin resistance of manure enterobacterales isolates of 26.2% in a study carried out in Thailand by Boonyasiri *et al*., (2023). Variation in results can be due to regional differences in antibiotic usage pattern

The results indicate a notable prevalence of colistin resistance among Enterobacterales isolates from composted manure in Benin City, with specific resistance rates observed in *E. coli* (20%), *Proteus spp*. (100%) and *Providencia spp* (100%). Such emerging prevalence of colistin resistance have previously been demonstrated by Stefaniuk and Tyski (2019) and Binsker *et al*., (2022). Findings underscore the presence of colistin resistance in environmental isolates, which is concerning given colistin's status as a last-resort antibiotic for multidrug-resistant Gram-negative infections. The varying resistance rates among different species suggest differences in either the exposure to selective pressures or the inherent capacity of these species to acquire and maintain resistance genes.

E. coli, a frequent resident of the human and animal intestinal tract, serves as an indicator of faecal contamination in environmental samples. The observation that 20% of E. coli isolates exhibited colistin resistance underscores the risk of antimicrobial resistance genes being prevalent in environments influenced by human or animal waste, particularly when used as fertilizer. Colistin resistance to *Escherichia coli* occurs via mutations in the chromosome or the acquisition of mobilized colistin-resistance (mcr) as explained by Bastidas-Caldes *et al*., (2023). A moderate resistance of *E. coli* strains to colistin has also been reported by Zhang *et al*., (2021).

Klebsiella spp. in this study showed no resistance to colistin, contrasting with earlier concerns about their role as reservoirs for resistance genes within agricultural environments. High sensitivity of *Klebsiella spp*. to colistin in this study is corroborated by previous studies including that of Azam *et al*., (2021). Contrarily emergence of colistin resistance *Klebsiella spp*. has also been documented by other studies (Mobasseri *et al*., 2019; Narimisa *et al*., 2022). This discrepancy highlights the variability in resistance patterns across different settings and emphasizes the importance of localized surveillance.

Proteus spp. associated with urinary tract infections, especially in healthcare environments, showed a 100% resistance rate to colistin in the isolates examined. This finding is particularly concerning given the organism's potential to contribute to healthcare-associated infections. The complete resistance observed among Proteus isolates signals a critical need for monitoring and controlling the spread of colistin resistance in both environmental and clinical contexts. *Providencia*, though less commonly discussed in the context of antibiotic resistance studies, showed resistance in the single isolate found. This finding indicates that less prevalent genera can also harbour resistance, underscoring the complexity of antimicrobial resistance ecology and the necessity for inclusive surveillance efforts. The total resistance to colistin shown by *Providencia spp* and *Proteus spp* in this study can be as a result of an intrinsic resistance expression of these enterobacterales. This has been corroborated in the literature by Gogry *et al*., (2021) and Torres *et al*., (2021) where both studies isolated enterobacterales of natural resistance to colstin. Gogry *et al*., (2021) specifically explains that, intrinsic colistin resistance in these organisms usually involves modulation of a lipid bi-layer that decreases or removes early charge-based interaction with colistin through up-regulation of multistep capsular polysaccharide expression.

Despite *Klebsiella spp*. isolates showing no resistance to colistin, their complete lack of sensitivity to several other antibiotics mirrors the resistance pattern observed in E. coli isolates. This shared resistance pattern between *Klebsiella spp*. and E. coli to critically important antibiotics such as Imipenem and Augmentin is alarming, highlighting the potential for environmental reservoirs to facilitate the spread of resistant genes across different bacterial species. Conversely, the *Providencia spp*. isolate demonstrated a distinctive susceptibility profile, being resistant to commonly used antibiotics like Cefotaxime and Augmentin, yet retaining sensitivity to other tested antibiotics, including Colistin. This pattern indicates that while *Providencia spp*. may be treated effectively with certain antibiotics, the resistance to others presents a complex challenge for public health. The unique resistance and susceptibility patterns of *Providencia spp*. underscore the necessity for individualized treatment plans based on comprehensive susceptibility testing to combat infections effectively.

These observations underscore the significant role environmental reservoirs, such as composted manure, play in the dissemination of antibiotic resistance genes. The presence of resistance to crucial antibiotics among Enterobacteriales isolates from environmental sources signals an urgent need for interventions aimed at curtailing the transmission of resistant bacteria from the environment to human populations. This situation necessitates a multifaceted approach, including the prudent use of antibiotics in agriculture, enhanced surveillance of antibiotic resistance in environmental settings, and the development of robust infection control strategies to prevent the spread of resistant infections.

The observed resistance patterns to key antibiotics such as Imipenem (IMP) and Augmentin (AUG) among Enterobacteriales isolated have profound implications for both public health and antimicrobial stewardship. The resistance of environmental isolates to these critical antibiotics underscores the alarming potential for horizontal gene transfer between environmental and clinical pathogens. This poses a significant threat as it can lead to the emergence of multidrug-resistant infections that are increasingly difficult to treat. Given that Imipenem is often reserved as an antibiotic for severe infections caused by Gram-negative bacteria, its efficacy being compromised due to environmental reservoirs of resistance emphasizes the urgent need for a global response to antimicrobial resistance that transcends human medicine to include environmental and agricultural practices.

5.2 Conclusion

This study revealed a concerning prevalence of colistin resistance among Enterobacterales isolated from composted manure in Benin City, Nigeria, with resistance observed in key pathogens including *E. coli, Proteus spp* and *Providencia spp*. Colistin is not a drug of choice in this locality, so it will be difficult to treat when this resistance strain find themselves in human. These findings highlight the critical role of environmental reservoirs in the spread of antibiotic resistance and underscore the potential public health implications, particularly in the context of last-resort treatments for multidrug-resistant infections. The data contribute to the growing body of evidence that supports the need for integrated, One Health approaches to antibiotic stewardship, spanning human, animal, and environmental health sectors.

5.3 Recommendations

1. It is recommended that comprehensive surveillance of antibiotic resistance in agricultural settings be established to monitor trends over time.
2. The development and implementation of guidelines for antibiotic use in agriculture are suggested to minimize the selection pressure for resistant strains.
3. Strategies for the safe disposal or treatment of livestock waste should be developed to reduce the spread of antibiotic-resistant bacteria in the environment.
4. Public health education campaigns should be initiated to raise awareness about the risks associated with antibiotic resistance and the importance of antibiotic stewardship.
5. Research into alternative farming practices and natural antimicrobials that could reduce the reliance on antibiotics in agriculture is encouraged.

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APPENDIX I

Media

The media used includes commercially dehydrated products and laboratory prepared media.

Macconkey Agar (CM7, Oxiod, England)

Constituents

Peptone	20.0grams
Lactose	10.0grams
Neutral red	0.075 grams
Bile salt	5.0grams
Sodium chloride	5.0grams
Agar	5.0grams
Distilled water	1000ml

pH 7.4 ± 0.2 at 25°C

Preparation

- 52 grams of MacConkey agar powder was weighed and suspended aseptically in
- 1 liter of sterile distilled water and was allowed to dissolve for 10 minutes.
- It was sterilized by autoclaving at 121°C for 15 minutes.
- The agar was cooled at 50°C, mixed and then poured aseptically into the petri dish.
- It was allowed to set and stored at 4°C for 2 weeks.

Mueller Hinton Agar (LabMal, Academy)

Constituents

Casein hydrolysate	17.5 grams

Beef infusion	2.0grams
Starch	1.5grams
Agar	17.0grams
Distilled water	1000ml

pH 7.3±0.1 at 25°C

Preparation

- 38grams of Mueller Hinton agar powder was weighed and suspended aseptically in 1
- Litre of sterile distilled water and was allowed to dissolve for 10 minutes.
- It was sterilized by autoclaving at 121°C for 15 minutes.
- The agar was cooled at 50°C, mixed and then poured aseptically into the petri dish.
- It was allowed to set and stored at 4°c for 2 weeks.

Citrate Koser Medium (Himedia (M069) Laboratories, India)

Constituents

Sodium ammonium phosphate	1.5grams
Potassium dihydrogen phosphate	1.0grams
Magnesium sulphate	0.2grams
Sodium citrate	3.0grams
Bromothymol blue	0.016
Distilled water	1000ml

pH 6.7±0.2 at 25°C

Preparation

- 5.7 grams of sodium citrate powder was weighed and suspended aseptically to 1 liter of sterile distilled water.

- This was allowed to dissolve for 10 minutes and then mixed.
- Equal volume of the broth was dispensed into sterile bijou bottles.
- It was then sterilized by autoclaving at 121°C for 15 minutes.
- It was allowed to cool and then stored at room temperature.

Urea Agar Base (CM53, Oxford, England)

Constituents

Peptone	1.0grams
Glucose	1.0grams
Sodium chloride	5.0grams
Disodium phosphate	1.2grams
Pottasium dihydrogen phosphate	0.8grams
Phenol red	0.02grams
Agar	15.0grams
Distilled water	95ml

Ph 6.8 +_ 0.2 at 25 c

Preparation

- 2.4 grams of urea agar base powder was suspended in 95ml of sterile distilled water.
- It was heated to boil to dissolve completely.

- It was sterilized by autoclaving at 121°C for 15 minutes.
- It was cooled to 50°C, 5ml of sterile 40% urea solution was added aseptically and mixed.
- It was then dispensed into sterile bijou bottles and was allowed to set in a slanted position.
- It was then stored at 4°C for 2 weeks

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Peptone Water (CM9, Oxiod, England)

Constituents

Peptone	10grams
Sodium chloride	5.0grams
Distilled water	100ml

pH 7.2 +_ 0.2 at 25C

Preparation

- 10 grams of peptone and 5 grams of sodium chloride were weighed and added aseptically to 1 liter of sterile distilled water.
- It was mixed and dispensed into sterile bijou bottles.
- It was sterilized by autoclaving at 121°C for 15 minutes.
- It was allowed to cool and stored at room temperature.

Chemical Reagent

All chemicals used in this study were of analytical grade and they include;

Gram Stain Reagent

Constituents

Crystal violet	0.2grams
Distilled water	80ml
Lugols iodine	2.0grams
Distilled water	100ml
Acetone	95%
Neutral red	1.0grams
Distilled water	100ml

1gram of neutral red was dissolved in a small amount of water and was made up to 100ml.

Normal saline

Constituents

Sodium chloride	0.85grams
Distilled water	100ml

Kovac's reagent

Constituents

p-dimethylaminobenzaldehyde	5g
Alcohol	75ml
Hydrochloric acid (concentrated)	2.5ml

Oxidase Strip

Composition

Tetramethyl-p-phenylenediamine	1g
Distilled water	100ml

Preparation

1g of the powder was dissolved in 100m of sterile water. 250 filter paper strips was then added to absorb the solution and then dried in the oven and allowed to cool before use

BUFFERED PEPTONE WATER

Formula	Grams/litre
Peptone	10g
Sodium chloride	5.0g
Di-sodium phosphate	3.7g
Potassium dihydrogen phosphate	1.5g
Distilled water	

Preparation

15g of the powder was dissolved in 1litre of distilled water and gently heated to dissolve the medium completely. It was then dispensed into bijou bottles and sterilized by autoclaving at 1210C for 15mins and then cooled to room temperature

APPENDIX II

Materials

Slides

Cover slips

Grease pencil

Wire loop

Straight wire

Test tubes

Petri dish

MacCartney bottles

Forceps

Equipment

Microscope

Hot air oven

Incubator

Autoclave

Test tube showing MIC of 2

INFORMED CONSENT FORM

Dear Sir/Ma,

Permission To Include Your Facility/Site in a Research

Title of Research: PREVALENCE OF COLISTIN RESISTANT ENTEROBACTERALES ISOLATED FROM COMPOSTED MANURE IN BENIN CITY, NIGERIA.

Investigators: **(1) – PROF. (MRS). H. O. OGEFERE**

Medical Microbiology Department of medical Laboratory Science, University Of Benin, Benin City.

(2) -- EKPENISI DEBORAH

Department of Medical Laboratory Science, University of Benin, Benin City.

Institution and Contact Address: Department of Medical Laboratory Science,
School of Basic Medical Sciences,
College of Medical Sciences
University of Benin,
P.M. B 1154
Benin City,
Edo State.

Phone Number:09020143098

Email address:ekpenisideborah@gmail.com

Commencement Date of Research: February 2024

Proposed duration of Research: Two (2) Months

Financial Sponsors: Self-Sponsored Research.

Conflict of Interest: We declare that there is no conflict of interest

The purpose of Research: The specific objective is to determine the prevalence of colistin-resistant enterobacterales isolated from composted manure in Benin city, Nigeria.

Estimated number of participants:239 samples will be collected from atmost 10 different agricultural site or farms in Benin city, therefore number of participant is 10.

Procedure Involved in the Study: Collection of composted manure from several sites in Benin City, Nigeria which will be subjected to various microbiological analysis.

Research Design and Methods: The study is case control.

Benefits: No direct benefits to site owners, however, if there is a high prevalence of colistin-resistant bacteria found after testing, the owners of the sites will be made aware.

Risks: There is no risk associated with this study.

Compensation/Inducement: Participants will receive no financial compensation and will not be forced or induced to participate.

Statement of Voluntariness and Circumstance for Withdrawal: Participants are allowed to withdraw from the research at any stage and the withdrawal will have no adverse effect on them in any form.

Confidentiality of Participants: Information obtained would be treated with utmost confidentiality

STATEMENT FROM PARTICIPANT(S)

I have read the description of the research. I understand that my participation is voluntary. I know enough about the purpose, methods, risks and benefits of the research study to judge that I want to take part in it.

Participants' Signature ………………………… Date……………………….

Participants' Name/Or Number (If Applicable) ………………………………………………….

Witness' Signature (If any) ……………………. Date ……………………….

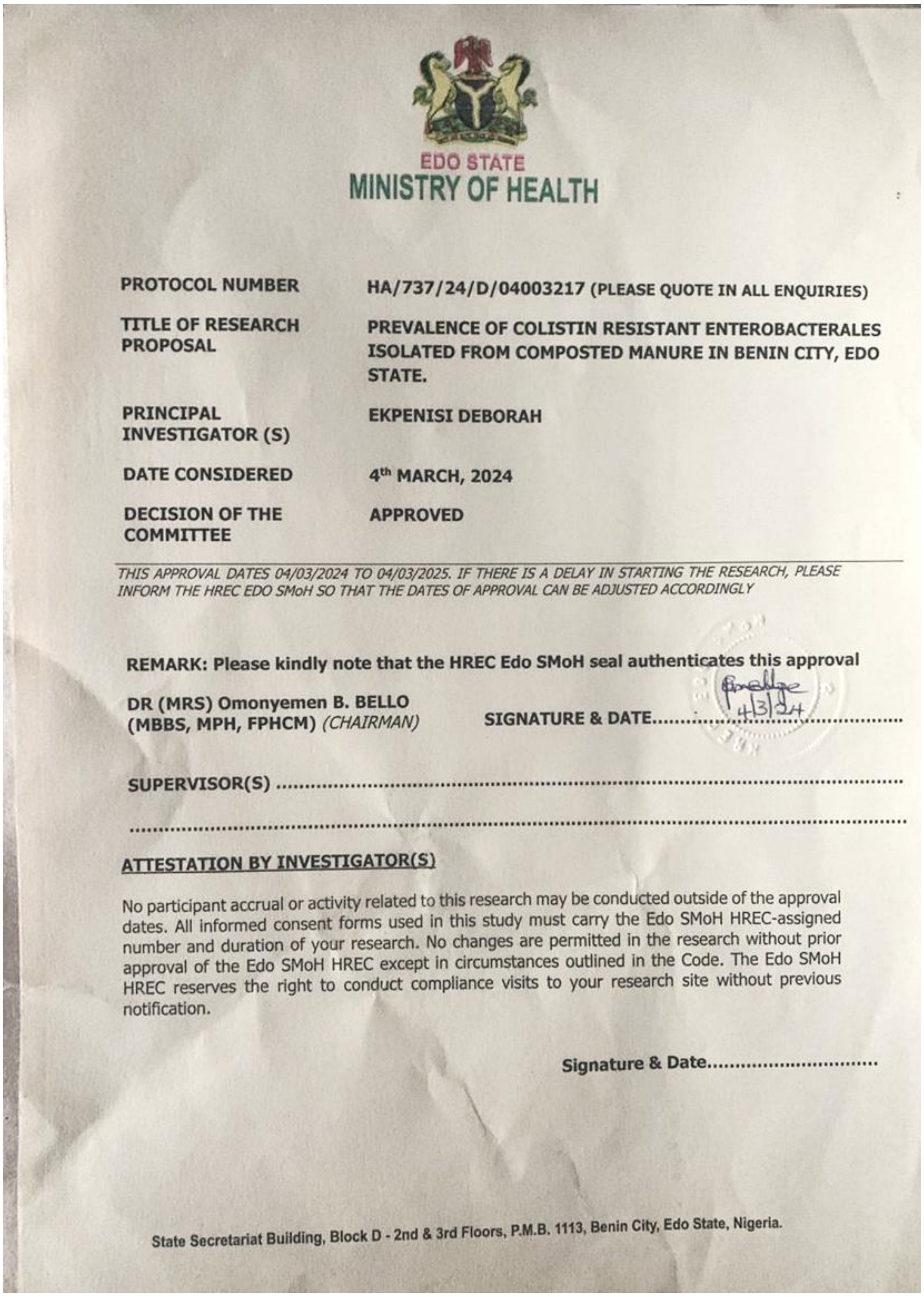
Witness' Name (If Applicable) ……………………………………………………………………

PLEASE KEEP A COPY OF THE SIGNED INFORMED CONSENT

For Official Use Only

Edo State ministry Health Approval Number…………………………………………

Commencement Date of Research: (dd/mm/yyyy) ………………………………



EDO STATE
MINISTRY OF HEALTH

PROTOCOL NUMBER	**HA/737/24/D/04003217 (PLEASE QUOTE IN ALL ENQUIRIES)**
TITLE OF RESEARCH PROPOSAL	**PREVALENCE OF COLISTIN RESISTANT ENTEROBACTERALES ISOLATED FROM COMPOSTED MANURE IN BENIN CITY, EDO STATE.**
PRINCIPAL INVESTIGATOR (S)	**EKPENISI DEBORAH**
DATE CONSIDERED	**4th MARCH, 2024**
DECISION OF THE COMMITTEE	**APPROVED**

THIS APPROVAL DATES 04/03/2024 TO 04/03/2025. IF THERE IS A DELAY IN STARTING THE RESEARCH, PLEASE INFORM THE HREC EDO SMoH SO THAT THE DATES OF APPROVAL CAN BE ADJUSTED ACCORDINGLY

REMARK: Please kindly note that the HREC Edo SMoH seal authenticates this approval

DR (MRS) Omonyemen B. BELLO
(MBBS, MPH, FPHCM) *(CHAIRMAN)* **SIGNATURE & DATE**............ 4/3/24 ....

SUPERVISOR(S) ....

....

ATTESTATION BY INVESTIGATOR(S)

No participant accrual or activity related to this research may be conducted outside of the approval dates. All informed consent forms used in this study must carry the Edo SMoH HREC-assigned number and duration of your research. No changes are permitted in the research without prior approval of the Edo SMoH HREC except in circumstances outlined in the Code. The Edo SMoH HREC reserves the right to conduct compliance visits to your research site without previous notification.

Signature & Date..............................

State Secretariat Building, Block D - 2nd & 3rd Floors, P.M.B. 1113, Benin City, Edo State, Nigeria.