AMERICAN UNIVERSITY OF BEIRUT

FIRST DETECTION OF THE PLASMID-MEDIATED COLISTIN RESISTANCE GENE, *MCR-1*, IN MULTIDRUG RESISTANT *E. COLI,* IN BACKYARD ANIMALS IN LEBANON

by
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Allah will exalt those who believe among you, and those who have knowledge,
TO HIGH RANKS.

ABSTRACT
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Ali Nawwaf Assi for Master of Science
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Title: First Detection of the Plasmid-Mediated Colistin Resistance Gene, *mcr-1*, in Multidrug Resistant *E. coli*, in Backyard Animals in Lebanon

The increase in antibiotic resistance has jeopardized the use of what is dubbed as last resort antibiotic, Polymyxin E. Specifically, the dissemination of mobile colistin resistance (*mcr-1*) gene has imperiled the effectiveness of this drug; posing a serious challenge worldwide.
Previous studies documented the occurrence of the mobile colistin resistance gene, *mcr-1*, that encodes colistin (Polymyxin E) resistance in *Escherichia coli* isolated from vital matrices such as irrigation, sea water, aquaculture, drinking water and broiler chickens in Lebanon. To investigate the spread of *mcr-1* gene to new matrices, we targeted backyard farms that have become a main food source for a large number of people in the country during the on-going economic crisis. In this study, 15 fecal samples were collected from a backyard farm in South Lebanon; 3 samples were collected from different animal species on the farm. All the samples were analyzed on agar media that was supplemented with 4 µg/ml colistin, and media without colistin to detect colistin and non- colistin resistant *E. coli.* Forty-five colonies were selected and purified from non-colistin plates, and twenty-seven colonies were selected and purified from colistin-containing plates. And all these colonies were selected for further analyses that included antimicrobial resistance phenotypes and gene-specific PCR analyses among others.
All the colonies that grew on colistin plates were *mcr-1* positive and were multidrug-resistant and the minimum inhibitory concertation of colistin varied between 4 and 1024 µg/ml. *E. coli* isolates (75%, 71%, and 44 %) carried bla_{TEM}, isolated from chickens, pigeons and sheep respectively, and only 25% isolates of the chickens carried $bla_{CTX\text{-}M}$.
Plasmid transformation experiments and sequencing for *mcr-1* gene were performed the transformants were colistin resistant and *mcr-1* positive, indicating that the gene was plasmid-borne. Detecting *mcr-1* positive *E. coli* and other resistant genes is a serious issue and can threaten the therapy of infectious diseases in the human and animal population.
Our findings emphasize the absolute need to tackle the surge of colistin resistance in Lebanon through a One Health approach.

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CHAPTER 1

INTRODUCTION

1.1. Antibiotics: General View

Antibiotics are compounds that are naturally produced by or obtained from microorganisms. They have the ability to deactivate or to destroy other microorganisms. Therefore, they have become pivotal tools in the battle against infectious diseases (1). Before introduction of antibiotics, bacterial diseases were considered as a significant threat to human and/or animal life. Even a simple tooth infection was considered as a life endangering disease. The discovery of antibiotics marked an important stage in the fight against bacterial infections, providing an effective solution to control bacterial diseases (4).

The story of antibiotics started in the year 1928 by the Scotish bacteriologist Sir Alexander Fleming who was, eventually, awarded the Nobel Prize in the year 1945 for this important discovery. Fleming cultured the bacterium *Staphylococcus aureus* and left the petri dishes in the laboratory for several days. Then, he observed that a common contaminant, a mold, grew on the same petri dishes, and possessed the ability to hinder the growth of the cultured bacteria (1).

This groundbreaking discovery marked the start of the “Golden Age of Antibiotics” in the mid-20th century. During this period, numerous antibiotics were isolated and characterized from various sources. Scientists such as Selman Waksman, Albert Schatz, and Dorothy Hodgkin played important roles in isolating and elucidating the structures of antibiotics like streptomycin and penicillin, paving the way for their mass production

and clinical application (2). During the 1960s, scientists intensified their efforts to discover novel compounds with the potential to fight pathogenic bacteria. For instance, a company dedicated its research to exploring quinine, a medication used against malaria, and successfully derived an antibacterial compound known as nalidixic acid. This particular compound exhibited the capability to control specific Gram-negative bacteria and emerged as an effective treatment option for managing urinary tract infections. Subsequently, this breakthrough laid the foundation for the development of fluoroquinolones, a notable class of antibiotics. As a result of the hard efforts exerted by scientists, many antibiotic classes were discovered and later introduced to the market, enriching the resources available for the treatment of bacterial infections (3).

The pharmacological actions of antibiotics involve the disruption of bacterial cells by either impeding their division or altering essential cellular functions or processes. Antimicrobial agents are traditionally categorized into two primary groups based on their effects on bacteria in laboratory settings: bactericidal and bacteriostatic. In general, bactericidal antibiotics are described as "killing" bacteria, while bacteriostatic antibiotics are believed to "inhibit the growth" of bacteria. (5).

Distinguishing between bacteriostatic and bactericidal drugs holds significant importance since static medications allow the bacteria to resume growth once the compound is eliminated from the body. However, the human immune system effectively reduces the pathogen count during an infection, making static agents like tetracycline effective treatments for certain diseases. Another important aspect is the mode of antibiotic action. For example, agents that induce the lysis of pathogens result in the release of toxic microbial molecules within the patient's body. These toxins can prolong the recovery period from the disease. Furthermore, the spectrum of antibiotic

activity is a key consideration, including whether the antibiotic is broad-spectrum or tailored for a specific pathogen species. In many scenarios, the causative pathogen is not always identified. Administering a broad-spectrum antibiotic allows for immediate and often cost-effective treatment without the need for additional diagnostic tests. However, for diseases necessitating prolonged treatment, such as tuberculosis, specialized agents that minimize effects on our normal bacterial flora are preferred. The advantages of narrow-spectrum agents extend beyond tuberculosis, and as rapid molecular diagnostic techniques become more convenient, their popularity is likely to increase.

Various antibiotic classes have the ability to selectively focus on certain cellular components and/or mechanisms within bacteria. So, we have protein synthesis inhibitors, DNA and RNA synthesis inhibitors, cell wall synthesis inhibitors, folate pathway inhibitors, and cell membrane inhibitors. (7)

1.1.2. 1.1.1. Anti-Microbial Resistance

Every living organism endeavors to adapt to its surroundings in order to ensure its survival. This process encompasses adapting to various factors such as food, water, oxygen levels, and even the presence of potentially harmful external elements. Consequently, it should come as no surprise that bacteria possess a notable capacity to persist and adjust to their surroundings. This adaptability includes the evolution of diverse resistance mechanisms against both established and novel antimicrobial agents. As a consequence, numerous bacterial strains have developed resistance, often exhibiting multidrug resistance, against these therapeutic agents. Therefore, these drugs

lose their effectiveness as preferred treatments for severe infections caused by these pathogens (8).

During his Nobel Prize acceptance speech, Sir Alexander Fleming expressed the following statement: "*Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant."* In spite Sir Fleming's warnings against the improper use of antibiotics, it has become increasingly clear that excessive misuse and abuse of these medications inevitably increase the rise of resistance. Thus, the swift emergence of antibiotic resistance poses a significant threat to public health and global animal husbandry practices (9).

Hence, as a definition, antimicrobial resistance (AMR) denotes the capability of microorganisms to persist and propagate despite the presence of chemical compounds, such as antibiotics, that are intended to inhibit their activity. AMR can arise through natural means or when bacterial cells are subjected to suboptimal concentrations of antibacterial agents. Given the appropriate circumstances, bacteria can acquire resistance to nearly any antibiotic, and in many cases, the mechanisms of AMR can be transmitted vertically or horizontally to offspring or other bacteria. Given the relatively rapid proliferation of bacteria, the dissemination of resistance can occur relatively rapidly and extensively.

1.1.3. Post Antibiotic Era

As reported by the World health organization (WHO), the rising issue of the antibiotic resistance has obtained global significance and is hindering the treatment of diseases caused by bacterial agents in humans.

Therefore, the prevalence of disease caused by multi-drug resistant agents is on a rise in both developed and developing countries, resulting in a significant increase in the number of affected patients. as Additionally, the cost of treating disease associated with AMR remains high. According to some authorities, it is expected that the global issue of the antimicrobial resistance (AMR) could lead to early deaths of approximately 100 million individuals by the year 2030, with an estimated 300 million individuals facing the same fate by 2050 (10). During a health pandemic, antibiotic resistance can have far-reaching consequences. It can complicate the management of bacterial co-infections that may arise alongside viral infections as well as increase the severity of secondary infections. Patients who are already critically ill or immunocompromised are particularly vulnerable to antibiotic-resistant infections, which can further strain healthcare resources and intensify the burden on healthcare systems. For example, aside from the direct impact of SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) infections, the surge in antimicrobial resistance poses an additional challenge to the ongoing COVID-19 (coronavirus disease 2019) pandemic. This marked by an increase in the prevalence of multidrug-resistant organisms (MDROs) secondary infections, including strains such as extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae*, carbapenem-resistant New Delhi metallo-β-lactamase (NDM)-producing *Enterobacterales*, *Acinetobacter baumannii*, methicillin-resistant *Staphylococcus aureus (*MRSA*),* pan-echinocandin-resistant *Candida glabrata*, and multi-triazole-resistant *Aspergillus fumigatus*. The underlying causes of this phenomenon are multifaceted, with a significant contribution arising from the extensive use of antimicrobial agents in COVID-19 patients, and this will add to what we have previously discussed regarding how far AMR can affect the health system (11).

From an economic perspective, between 2014 and 2050, the world is anticipated to suffer a loss of economic output amounting to approximately $ 60 to $ 100 trillion, equivalent to one year of the global economic output. Additionally, it is expected that the gross domestic production could witness a decline of 2% to 3.5% by the year 2050 (10).

The expansion of antibiotic resistance is expected to worsen poverty levels mainly in low-to middle-income countries; primarily through prolonged hospitalizations, increased treatment expenses, and premature deaths, all of which directly hinder overall productivity rates. In the United States of America alone, the antimicrobial resistance crisis carries a substantial economic burden. Estimates suggest that the healthcare sector faces costs of up to $20 billion, while productivity loss accounts for approximately $35 billion. Furthermore, research conducted by the World Bank indicated that antimicrobial resistance is likely to intensify poverty levels, exerting serious effects on the most impoverished nations. (12)

Additionally, the increase of the antimicrobial resistance would result in 11% decrease in the production of livestock by 2050.This might complicate the economic challenges due to the financial losses in animal production mainly in low and middle-income countries, as stated by the World Bank.

As resistance to antibiotic rises within the agricultural sector, the efficiency of these antibiotics on livestock will significantly decrease, resulting an increase in the infection rates and the spread of infections. This will cause disruptions in the animal mortality and morbidity rates. This will also cause a drop in the production and trade of livestock animals. Consequently, a spike in the prices of protein sources such as meat and eggs is expected. This will result in shortage of these nutritional sources. This is an alarming

situation taking into consideration the growing global need for the animal-based protein to meet the demand of an increasing world population (12).

1.1.4. Backyard Animals and Antibiotics

Backyard animals play and important role in the lives of people, mainly those living in low and middle-income countries. This is because backyard animals such as chicken, goats and pigs provide a source of income and a consistent source of protein-rich food. Subsequently, small-scale farming of livestock contributes in providing improved nutrition and dietary diversity such as vitamins, minerals and high-quality proteins, especially where access to reasonably priced animal protein is limited (18).

This make backyard animals a notable contributor in the household food security. Furthermore, backyard animals play a vital role in the resilience of poor communities (21).

Backyard animals also represents a safety net during economic crisis, allowing households to cope with uncertainties (18). Being a source of income in poor and middle-income countries, backyard animals act as a poverty reduction element. They can be an alternative livelihood for rural communities, mainly those engaged in subsistence agriculture. As this income can be used in the coverage of basic needs, like education and healthcare expenses in addition to other income generation activities (20).

Lebanon, a country in the Mediterranean region, has a rich tradition of backyard animal husbandry. Including different species such as goats, pigeons, cows, pigs and chickens. These animals have an important role in the economic landscape and agriculture of Lebanon, being a source of food such as dairy and meat products (23). Goats and sheep are widely used in backyard farming. They give valuable source of milk, meat and

leather. Goat and sheep milk are prevalently consumed and used in the production of different local dairy products (24).

Chickens are also raised in backyard settings, serving as a valuable source of meat and egg. Their adaptability, low cost of maintenance, and high productivity make them a popular choice among farmers and families (25).

Cows are primarily kept for milk production, thus contributing to the local dairy industry. Cows also play a role in small-scale farming for meat consumption in certain regions (24). Pigeons are raised for meat and as pets too in Lebanon. This type of animal husbandry is referred to as “pigeon fancying,” which is a traditional practice with cultural significance. Pigeon meat and eggs are highly valuable and are often eaten during special events (25). Pigs are also in backyard settings; although they are less common compared to other animals, mainly due to religious aspects. However, they provide a source of meat and contribute to local pork production (26).

Veterinary antimicrobial products have been extensively administered to livestock for treatment, prevention of infection, and growth promotion (27). In 2010, approximately 63,151 tons of antibiotics were used in global food-animal production. The quantity is expected to increase by 67%, reaching a total of 105,596 tons in 2030 (30). The use of antimicrobials as growth promoters and disease prophylaxis is widespread among food animals in low and middle-income countries.

In the United States, approximately 80% of the nation's yearly antimicrobial consumption was attributed to the use of antimicrobials in food animals (31). It is worth noting that a substantial portion of these antimicrobials are essential in human medicine, for treating common infections and carrying out critical medical procedures like major surgeries, organ transplantation, and chemotherapy (32).

A significant proportion of animals raised for food production receive medication throughout their lifespan. However, the use of these drugs raises concerns regarding the presence of residues in edible tissues, posing potential toxicological risks to consumers. These residues can have adverse effects such as allergic reactions, disturbances in intestinal flora, and the emergence and dissemination of drug-resistant bacteria (28). Consequently, the European Union (EU) banned the nontherapeutic use of antimicrobials in animals for growth promotion. This regulation was implemented by EU through Regulation No 37/2010 in 2010, which gives Maximum Residue Limits (MRLs) for biologically derived consumable substances such as meat and milk so that consumer safety is minimized (29).

The extensive use of antimicrobials in livestock plays a role in the emergence of antimicrobial-resistant bacteria (ARBs) through selection (32). Therefore, this has tremendous public health implications, because ARBs from animals could be passed on to humans via various ways including environment, food products and direct exposure to farmers (33). While establishing direct linkage is challenging due to the ecological nature of antibiotic selection pressure, however studies have consistently shown a strong association between the prevalence of AMR in animals and humans, as well as the levels of antimicrobial use in animals and the prevalence of AMR in both animal and human populations. In European countries, researches indicated an important relationship between consumption of different classes of antimicrobials and occurrence of antimicrobial-resistant commensal *Escherichia coli* in livestock (34). Furthermore, there is evidence suggesting that repeated exposure to low doses of antimicrobial agents as observed during the administration of growth-promoting antimicrobials and

prophylactics create conditions favorable for the emergence and spread of AMRs among animals (32).

The problem is worse in small-scale animal production in low-and middle-income countries, since these farmers commonly rely on information and misinformation shared by vendors of veterinary products when it comes to using antibiotics. However, due to limited access to veterinarians, these producers often turn to advice from family members and neighbors for guidance in their decision-making process. (35) In addition to the uncontrolled access to antibiotics in veterinary stores and especially the access to critically important antibiotics, like colistin. According to a study done in Lebanon, the availability of twelve different colistin drug brands in the market without the need for a veterinarian's prescription raises concerns about uncontrolled access to this vital antibiotic. These drugs, are commonly used in the treatment and prevention of diseases in poultry. The packaging of these drugs often lacks clear and specific treatment instructions, with indications that are ambiguous, nonspecific, or cover a wide range of diseases. While none of the packages explicitly support the use of these drugs for growth promotion, there are anecdotal reports of their sale for such purposes. This unregulated access to colistin higher the potential risks in terms of appropriate usage and the development of antibiotic resistance. (36)

1.1.5. Colistin- The Last Resort Antibiotic

Antibiotics are arranged into different classes according to their chemical structure and mechanism of action. Each class targets specific types of bacteria or inhibits particular cellular processes (13). One commonly used class is the beta-lactam antibiotics. These antibiotics interfere with bacterial cell wall synthesis, by acylating the

transpeptidase enzyme responsible for cross-linking peptides and forming peptidoglycan, leading to cell death. Cephalosporins represent the most extensive category within the beta-lactam antibiotics class and are widely regarded as the initial choice for treatment. (14)

Cephalosporines have been divided into four different generations. The first-generation cephalosporins exhibit superior activity against gram-positive bacteria but are less effective against gram-negative bacteria. In contrast, the second-generation cephalosporins are more effective against gram-negative bacteria but have limited activity against gram-positive bacteria. The third-generation cephalosporins demonstrate enhanced activity against gram-negative bacteria and are more resistant to beta-lactamase enzymes compared to the first and second generations. The fourth-generation cephalosporins are an improved version of the third generation, possessing increased stability against beta-lactamase enzymes and better efficacy against gram-positive bacteria. (14) Nevertheless, with the emergence of cephalosporin resistance, an alternative treatment has been employed, carbapenems. Carbapenems hold a vital position in our implementation of antibiotics, offering a crucial range of action and exceptional potency against both Gram-positive and Gram-negative bacteria among the numerous β-lactam agents available (16). Regrettably, the recent appearance of multidrug-resistant (MDR) pathogens poses a significant threat to the effectiveness of this essential category of life-saving medications, driving the usage of colistin, the last resort antibiotic. (17)

Discovered in the 1940s by Koyama in Japan (41), the polymyxin group of polypeptide antibiotics represented a significant breakthrough in combating Gram-negative bacteria. It is a unique group of cyclic oligopeptide antimicrobials, comprising five chemically

distinct compounds: polymyxins A, B, C, D, and E (41). Among these antibiotics, colistin (polymyxin E) and polymyxin B emerged as the primary options used in clinical settings. (37) Colistin and polymyxin B exhibit a minor variation in their peptide ring, differing by a single amino acid. Polymyxin B contains phenylalanine, while colistin features leucine Polymyxin B is directly administered as an active antibiotic, whereas colistin is administered in the form of an inactive prodrug known as colistin methanesulfonate, also termed colistimethate (CMS) (42).

Originally discovered in the Gram-positive bacterium Paenibacillus polymyxa (38) Polymyxins are a unique class of cyclic-peptide antibiotics synthesized through non-ribosomal means, featuring a hydrophobic tail (39). The chemical composition of polymyxins bears resemblance to cationic antimicrobial peptides (CAMPs) such as defensins and gramicidin. Polymyxins, being cationic polypeptides, consist of a cyclic heptapeptide with a tripeptide side chain that is acylated by a fatty acid tail at the N-terminus. The inherent toxicity of colistin can be attributed to the hydrophobic nature of the N-terminal fatty acyl segment, which also significantly contributes to its antimicrobial activity. (42).

Polymyxins exhibit potent activity against Gram-negative pathogens. The mechanism of action involves disrupting membrane permeability through a combination of polar and hydrophobic interactions. Notably, an electrostatic interaction occurs between the positively charged residues of polymyxin and the negatively charged lipid A moieties of lipopolysaccharides (LPS), which are anchored on the outer leaflet of the bacterial membrane (40). The polymyxins also exert their action by inhibiting essential respiratory enzymes, specifically targeting type II NADH-quinone oxidoreductases (NDH-2) present in the inner membrane of bacteria (43).

Although the nephrotoxicity and neurotoxicity potential of polymyxin E (colistin) have hindered its widespread adoption in regular clinical therapies, the emergence of multidrug-resistant (MDR) or extremely drug-resistant (XDR) Gram-negative bacteria has prompted a renewed interest in colistin as a last-resort treatment for severe bacterial infections (37).

The administration of colistin did not stop in human medicine, it is also been extensively used in veterinary over the course of numerous decades, they have been utilized in the management and prevention of infectious diseases (44). Another application involves promoting growth, which is a widely practiced phenomenon globally (44).

1.1.6. Colistin- Resistance Mechanisms

1.1.6.1.Intrinsic Resistance.

Certain Gram-negative species, naturally exhibit resistance to the activity of colistin. In the majority of instances, the observed resistance to colistin is primarily attributed to alterations in the structure of lipid A. This modification results in a reduction or complete elimination of early charge-based interactions with colistin, predominantly due to the up-regulation of multistep capsular polysaccharide expression (45). For example, *Vibrio cholera*, *Proteus spp*., *Providencia spp*., *Morganella morganii*, *Brucella, Campylobacter*, and *Neisseria spp.* (46).

1.1.6.2.Acquired Resistance

Chromosomal Mutation: Colistin has been heavily employed in animal farming and veterinary practices, leading to the development of resistance to colistin mediated, by both chromosomal and plasmid mechanisms. Bacteria *like Klebsiella pneumoniae*,

Escherichia coli, *Pseudomonas aeruginosa*, *Salmonella* and *Acinetobacter baumannii* have shown acquired resistance to polymyxins through changes in their genetic structure (chromosomal mutation). These changes, particularly in the structure of lipopolysaccharides (LPS), alter the charge of LPS and reduce the ability of polymyxins to bind effectively to the outer membrane, leading to resistance. (47)

The way bacteria become resistant to colistin is through alterations in their genetic structure, alike to how certain bacteria are naturally immune to colistin. (45)

A range of molecular mechanisms has been identified, with the predominant alterations taking place through the addition of cationic groups, namely 4-amino-4-deoxy-l-arabinose (L-Ara4N) and phosphoethanolamine (pEtN), to the lipid membrane of bacterial strains. (48) The processes of modifying lipopolysaccharides (LPS) in bacterial strains significantly involve the intricate functions of two-component systems, namely *PhoPQ* and *PmrAB*. These systems play a pivotal role by facilitating the addition of cationic groups to the LPS membrane, leading to substantial alterations in the bacterial cell structure. (49)

Plasmid-Mediated: The occurrence of colistin resistance, specifically through plasmid-mediated mechanisms, is closely associated with the existence of mobile colistin resistance genes (*MCR*) within the bacterial genetic makeup. These genes, found on plasmids, play a fundamental role in conferring resistance to colistin. In November 2015, Liu and colleagues reported the initial instance of colistin resistance transferred through plasmids, specifically involving the *mcr-1* gene, within *Enterobacterales* originating from both animals and humans. (47) *E. coli* SHP45, which was isolated from a pig in China, served as the focal point of this study. The research unveiled that this particular gene was hosted on a plasmid, making it susceptible to horizontal transfer

between bacteria. Furthermore, the gene demonstrated the alarming potential to spread across international borders, posing a significant global threat to the management and prevention of infectious diseases. (50) Colistin operates by engaging in electrostatic interactions with lipid A present in bacterial cell membranes. However, the presence of *mcr-1* can diminishes this interaction, consequently undermining the effectiveness of colistin and facilitating the development of resistance in bacteria. (51)

Before the revelation of the mobile colistin resistance gene, colistin resistance was assumed to be predominantly driven by chromosomal mutations, with limited lateral transfer of resistance among bacteria. However, in the current scenario, the *mcr-1* gene has been identified in numerous countries across five continents. (52)

Lebanon, a country characterized by lax regulations and insufficient enforcement regarding the appropriate use of colistin in agricultural contexts, experiences a notable prevalence of *mcr-1*, particularly within the agricultural sector. Though researches are limited however reports have been published about the presence of *mcr-1* in various sources, including irrigation water, poultry feces samples, drinking water, well water, sewage in refugee camps, seawater, human samples, and aquaculture (53-56). These studies, though it is still limited, pushed the Lebanese parliament to publish a decree under the number 221 in the official governmental newsletter to ban the importing of veterinary drugs that contain colistin.

1.2. Conclusion

In Lebanon, where the economic crisis has posed additional challenges, backyard animals play a vital role in sustaining livelihoods and community health. They offer a dependable source of income, promoting self-sufficiency and resilience, besides being a trusted, safe, and organic food source. The variety of animals, such as poultry, goats,

sheep, pigs, pigeons, and cows, helps address concerns about food security by providing protein sources at the household level. However, the use of antibiotics in animal farming, particularly colistin, which is widely available and extensively used, raises concerns. Since the initial identification of *mcr-1* in 2015, the global health community has been deeply concerned. In Lebanon, there have been no reports about the dissemination of *mcr-1* positive MDR bacteria in backyard farms that lack proper practices and sanitation. Making backyard animals, especially with their interaction with each other, farmers, agricultural lands, and water bodies, a high potential source for the dissemination of MDR bacteria and the mobile colistin resistance genes including *mcr-1*. Hence, the work outlined in this thesis has focused on the detection of *mcr-1* gene in backyard animals in Lebanon.

CHAPTER 2

METHODOLOGY

A backyard farm was selected in South Lebanon based on two criteria: 1- availability of multiple species in close proximity, and 2- accessibility to researchers. The farm was labeled as farm1.

Farm 1 consisted of approximately 200 pigeons, 20 cows, 220 goats, 14 sheep, and 20 chickens. The following is some information about this farm: This backyard farm is considered a source of money for a 7-member family. The animals are used for milk, egg, and meat production for the nearby stores and a small dairy plant in that region. Additionally, the manure from this farm is distributed and used as fertilizers to the adjoining orchards and agricultural lands.

The animals in this farm are fed from the weeds in the neighboring lands and/or imported feeds. The source of water is from the domestic irrigation water. The owner of farm one declared the use of only 3 types of medicines for cattle: 1- Ivotek® 2- Alfameca1® 3- Tylokel (20) ®. The farm showed a lack of appropriate hygienic practices.

2.1. Sample Collection

Fifteen fecal samples were collected from a backyard farm, in which 3 samples were collected from each animal species.

For pigeons, chickens, and cows, the fresh fecal samples were taken via sterile swabs, but for sheep and goats, fresh fecal droppings were collected. The samples were put in a sterile 15 ml falcon tube and were placed immediately on ice. Further analysis was conducted on the same day of sample collection.

2.2. Samples preparation and Culturing

Two ml of Peptone Buffer Water (PBW) was added to each tube containing the fecal sample which was suspended by vortexing for about 30 seconds. 50µl were taken and spread on RAPID' E. coli 2 Agar supplemented with 4 µg/ml colistin. As well as 20µl were spread on RAPID' E. coli 2 Agar without colistin.

One ml from each sample was transferred to another 15 ml tube and 0.5 ml glycerol 80% was added to each tube and then those were stored at -80°C.

The plates were then incubated at 37°C for 24 h, 1~3 colonies from each sample were isolated and further purified. Then, the purified isolates were placed in 1 ml Luria-Bertani (LB) broth with 0.5 ml glycerol 80% and stored at -80°C for further analysis.

2.3. Antimicrobial Resistance (AMR)

All the *E. coli* isolates were subjected to antimicrobial analysis using the disk diffusion method. The optical density of each colony was adjusted to 0.05 (wave-length: 600 nm) in Mueller-Hinton (MH) broth. Sterile cotton swabs were used to streak the cultures on Mueller-Hinton agar (MHA) plates. Eighteen different commercially-available antibiotics disks were tested against each culture, four antibiotics per plate, which was incubated at 37°C for 18-24 hours. The antibiotics were: Penicillin (PEN), ampicillin (AMP), amoxicillin+calvulanic acid (AMC), cefepime (FEP), cefotaxime (CTX), cephalexin (LEX), cefixime (CFM), doripenem (DOR), imipenem (IPM), meropenem (MEM), gentamicin (GEN), kanamycin (KAN), streptomycin (STR), tetracycline (TET), ciprofloxacin (CIP), norfloxacin (NOR), trimethoprim-sulfamethoxazole (SXT), and chloramphenicol (CHL). The antibiotics in the resistance profile were organized according to the order of antibiotics/ classes listed in the Clinical

and Laboratory Standards Institute CLSI guidelines. The diameter across each disk was measured and assessed according to the CLSI standards to diagnose whether the colony is resistant, with intermediate resistance, or susceptible.

2.4. Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) for colistin was done on all *mcr-1*-positive-*E. coli*, based on CLSI recommendations, to determine the susceptibility/resistance profile against colistin. After adjusting the optical density of each colony to 0.05 (wave-length: 600 nm), the cultures were challenged with a serial dilution of colistin in 96-well plates. The concentrations of colistin ranged between 1 µg/ml to 1024 µg/ml. The plates were incubated for 24 hours at 37°C, and then the plates were analyzed with an Infinite M200PRO microplate reader at $\lambda = 600$ nm. The results were compared with the reading of control wells.

2.5. Plasmid Extraction and Transformation

Plasmid extraction was first done for 12 *mcr-1* positive isolates by using the QIAGEN® Plasmid Mini Kit according to the manufacturer procedures. The extracted plasmids were stored at -20°C for further analysis.

Secondly, chemically-competent *E. coli* JM109 was used as a receiver for the extracted plasmids. The competent cells were placed on ice for 10 min. Next, 0.05 ml of the competent cells were added to 10 µl of the purified plasmid and incubated on ice for 30 min. The mixture was settled in a water bath at 42°C for 2 min followed by 90 sec. on ice. 940 µl of LB broth was added to the mix and then incubated in the shaker at 37°C for 1hr 45 min. The mixture was then centrifuged for 1 min and 0.9 ml was discharged.

The pellet was resuspended and spread on RAPID' E. coli 2 Agar with 2 µg/ml colistin. The plates were incubated at 37° C for 24 hours, and bacteria colonies (transformants) were taken and were stored with 1ml LB broth and 0.5ml 80% glycerol in cryovials at -80°C for further analysis.

2.6. Polymerase Chain Reaction (PCR)

The PCR reaction used genomic DNA extracted from bacterial colonies through a boiling process. One to two bacterial colonies were suspended in 100 µl of DNase-free water and subjected to a 15-minute incubation at 95°C to ensure complete cell lysis. After centrifugation at 14000 rpm for 2 minutes, the supernatant, which contains the genomic DNA, was carefully collected and transferred to a new sterile tube. The obtained DNA was then stored at -20 °C.

Detection of *mcr-1* gene was done using Polymerase Chain Reaction (PCR). The DNA extracted was employed for screening the *mcr-1* gene, utilizing specific CLR5 primers (CLR5-F 5′-CGGTCAGTCCGTTTGTTC-3′ and CLR5-R 5′-CTTGGTCGGTCTGTA GGG-3′). The PCR reactions were prepared by adding 3 µl of genomic DNA to a 20 µl PCR reaction tube, which included 0.5 µl each of the forward and reverse primers, 4 µl of master mix (5x FIREPol® Master Mix Ready to Load), and 12 µl of DNase-free water. The PCR analysis comprised 38 cycles in a thermal cycler (VWR), involving denaturation at 95°C for 1 minute, primer annealing at 56°C for 45 seconds, and an extension cycle at 72°C for 1 minute, with a final extension for 10 minutes at 72°C The resulting *mcr-1* gene fragment had a size of 309 bp. The amplified gene product was loaded onto a 1% agarose gel stained with ethidium bromide and subjected to

electrophoresis for 35 minutes at a constant voltage of 100V. Visualization was performed using the GelDoc reader in the gel imaging system.

Detection of the antimicrobial resistance genes: PCR analysis was employed to detect the presence of beta-lactam genes, namely *blaTEM*, *blaCTX-M*, *blaSHV*, *blaKPC*, *blaIMP, blaNDM, blaOXA-48*, and Class 1 Integron, using specific primers outlined in Table 1. The PCR reactions were assembled by combining 3 µl of DNA template, 0.5 µl each of the designated forward and reverse primers, 4 µl of Master Mix (5x FIREPol® Master Mix Ready to Load), and 12 µl of DNase-free water. The PCR conditions are detailed in Table 1, and the reactions underwent 38 cycles in a thermocycler. Subsequently, the resulting gene amplicons were observed by electrophoresis on a 1% agarose gel stained with ethidium bromide, running for 35 minutes at 100V.

Table 1 shows details about the tested genes and their PCR conditions.

Gene and Primers	**Denaturation**	**Annealing**	**Extension**
mcr-1 gene; size: 309 bp CLR5-F (5′-CGGTCAGTCCGTTTGTTC-3′) and CLR5-R (5′-CTTGGTCGGTCTGTA GGG-3’)	95°C for 1min	56°C for 45 sec	72°C for 1 min
bla-TEM ; size: 953 bp Forward: ACCAATGCTTAATCAGTGAG Reverse: GCGGAACCCCTATTTG	95°C for 1min	56°C for 45 sec	68°C for 1 min
bla-CTX-M1-group ; size:804 bp Forward: ATGTGCAGYACCAGTAARGTKATGGC Reverse: TGGGTRAARTARGTSACCAGAAYCAGCGG	95°C for 1min	56°C for 45 sec	68°C for 1 min

bla-SHV; size: 822 bp Forward: CACTCAAGGATGTATTGTG Reverse: TTAGCGTTGCCAGTGCTCG	95°C for 1min	56°C for 45 sec	68°C for 1 min
bla-KPC ; Size: 538 bp Forward: (CATTCAAGGGCTTTCTTGCTGC) Reverse: (ACGACGGCATAGTCATTTGC	95°C for 1min	56°C for 45 sec	68°C for 1 min
bla-OXA-48; Size: 281 bp Forward: (GCTTGATCGCCCTCGATT) Reverse: (GATTTGCTCCGTGGCCGAAA)	95°C for 1min	56°C for 45 sec	68°C for 1 min
bla-NDM; size: 621 bp Forward: GGTTTGGCGATCTGGTTTTC Reverse: CGGAATGGCTCATCACGATC	95°C for 1min	56°C for 45 sec	68°C for 1 min
bla-IMP ; size: 740bp Forward (TGAGCAAGTTATCTGTATTC) Reverse: (TTAGTTGCTTGGTTTTGATG)	95°C for 1min	56°C for 45 sec	68°C for 1 min
Class 1 gene; size: Various Forward: GGCATCCAAGCACAAGC Reverse: AAGCAGACTTGACTGAT	95°C for 1min	56°C for 45 sec	68°C for 1 min

Box-PCR: Repetitive sequence-based PCR typing using the BOX-A1R primer (5'-CTACGGCAAGGCGACGCTGACG-3') was conducted on all *mcr-1* positive isolates to assess their genotypic diversity. Each PCR reaction, performed in a 25 µl PCR reaction tube, included 3 µl of DNA template, 0.5 µl of Box-A1R primer, 4 µl of Master Mix (5x FIREPol® Master Mix Ready to Load), and 17.5 µl of DNase-free water. Amplifications followed a specified protocol: an initial denaturation step lasting 2 minutes at 94°C, then 38 cycles consisting of 30 seconds at 94°C, annealing at 50°C for 1 minute, and extension

for 8 minutes at 65°C. The final elongation occurred for 8 minutes at 65°C. The generated fingerprints were visually interpreted and subjected to analysis on a 2% agarose gel stained with ethidium bromide, electrophoresed at 100 V for 75 minutes.

The plasmid types were identified using PCR Based Replicon Typing Kit 2.0 (Diatheva) as described by the manufacturer. The visualization of the amplified DNA product was done by inoculating the amplified product into 2.5% agarose gel with ethidium bromide and was run for 45 mins at 100mV until the amplicons generated by the multiplex reactions are well distinguished. Subsequently, the gel was positioned in the imaging system, and photographs of the gels were taken.

2.7. Sequencing

Commercial sequencing was done after purifying the amplified genes with QIAquick® PCR Purification Kit and QIAquick® Gel Extraction Kit.

CHAPTER 3

RESULTS

Fifteen fecal samples were collected from a backyard farm in South Lebanon, in which 3 fresh fecal samples were collected from each animal species (Figure 1). Twenty-seven colonies were selected from colistin supplemented plates (figure 2), and then screened for the detection of the *mcr-1* gene using specific CLR5 primers. Forty-five colonies were collected from non-colistin plates to be analyzed for antimicrobial screening.

Figure 1 The targeted backyard farm

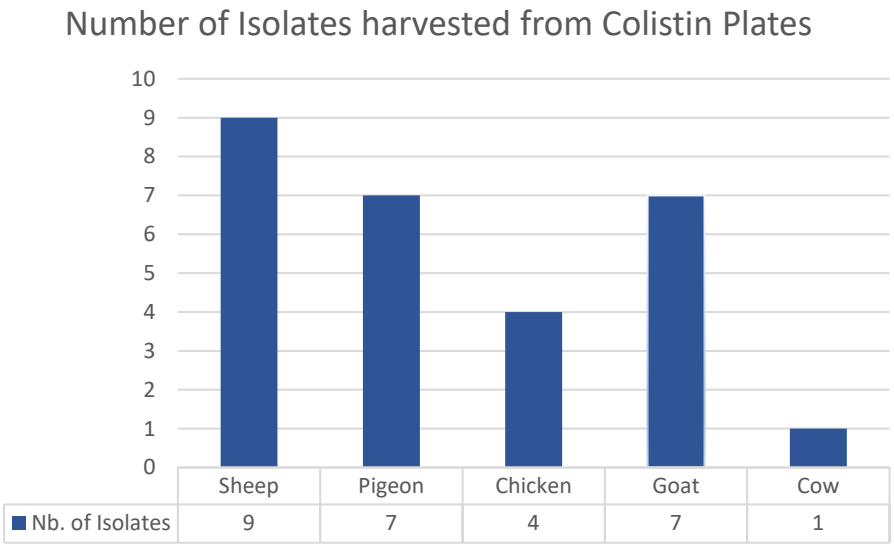

Figure 2 Number of colistin resistant *E. coli* that were collected from each animal species

The *mcr-1* gene was detected in all the colistin-resistant *E. coli* strains in the 5 different animal species (Fig 3).

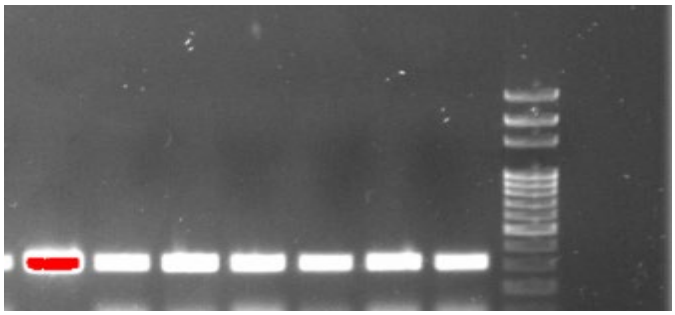

Figure 3 Agarose gel of the amplified *mcr-1* gene fragment (309 bp), with the DNA Ladder

Colistin's minimum inhibitory concentration (MIC) was ≥ 4 µg/ml for all the *mcr-1* positive isolates confirming the colistin resistance (Figure 4).

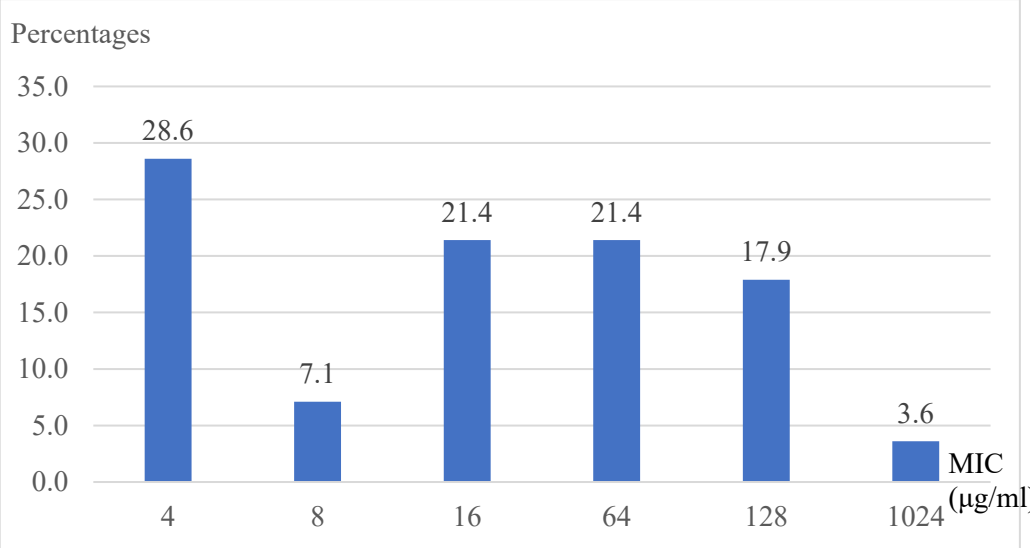

Figure 4 Percentages of Colistin's Minimum Inhibitory Concentration (MIC) of the *mcr-1*-positive *E. coli* Isolates

For sheep, the MIC ranged between 4 µg/ml and 64 µg/ml. 56% had an MIC 4 µg/ml., 11% with MIC 8 µg/ml., 11% with MIC 16 µg/ml. and 22% had MIC 64 µg/ml. Figure (5)

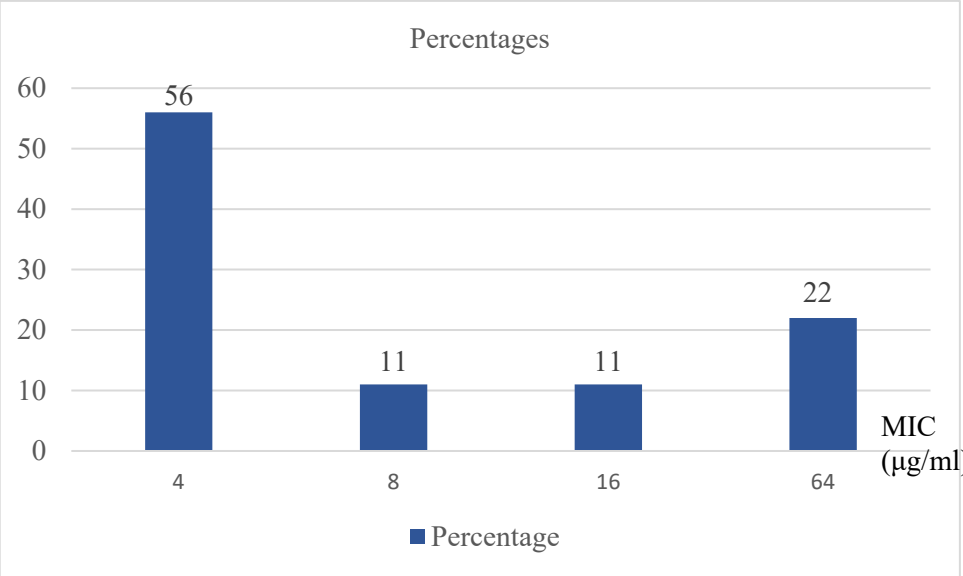

Figure 5. Percentages of Colistin's Minimum Inhibitory Concentration (MIC) of the *mcr-1*- positive-*E. coli* isolated from sheep fecal samples.

For pigeons, the MIC reported was between 8 µg/ml and 64 µg/ml. 14% had MIC 8 µg/ml, 43% had an MIC 16 µg/ml, and 43% also with MIC 64 µg/ml. Figure (6).

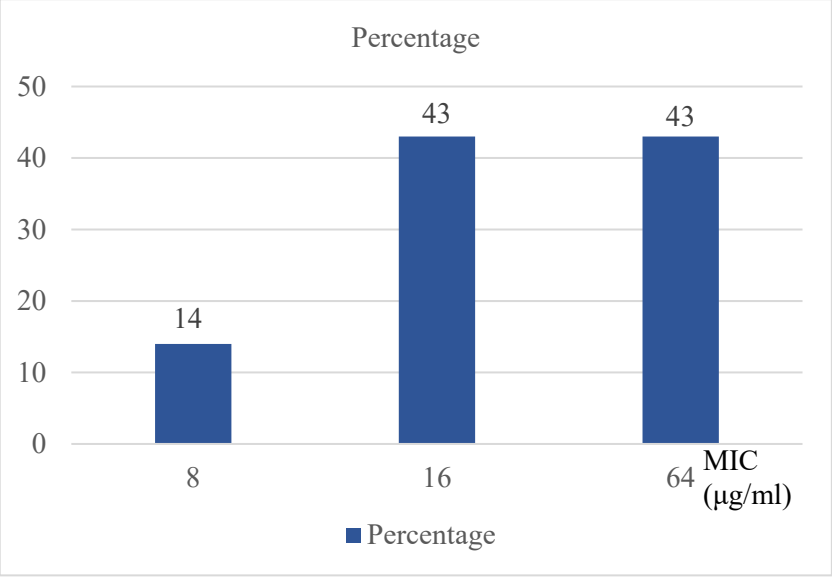

Figure 6. Percentages of Colistin's Minimum Inhibitory Concentration (MIC) of the *mcr-1*- positive-*E. coli* isolated from pigeons' fecal samples

For chicken, the MIC ranged between 4 µg/ml and 1024 µg/ml. And the values 4, 16, 128, 1024 µg/ml had all equal percentage (25%). Figure (7)

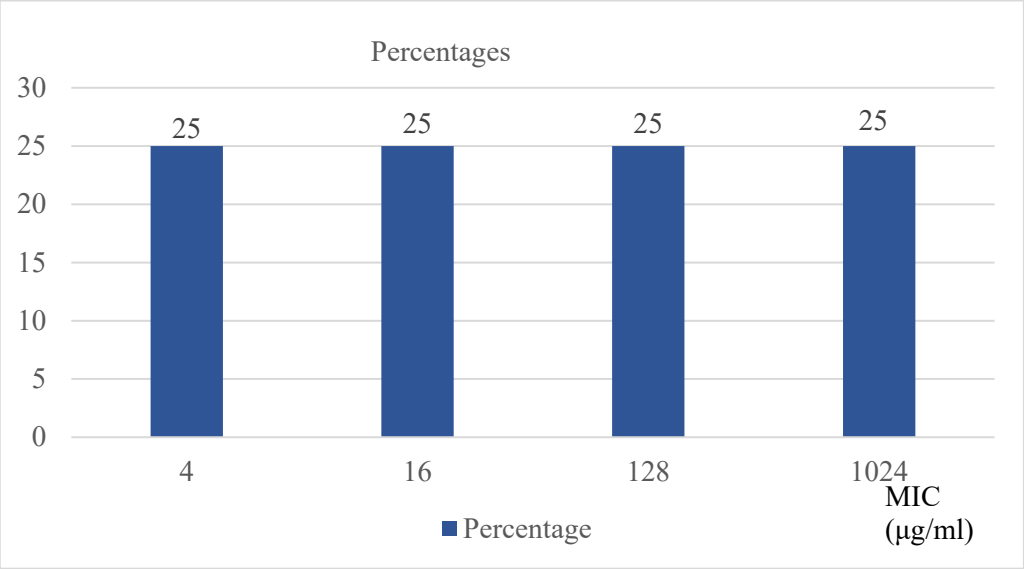

Figure 7. Percentages of Colistin's Minimum Inhibitory Concentration (MIC) of the *mcr-1*-positive-*E. coli* isolated from chicken fecal samples

For goats, the MIC ranged between 4 and 128 µg/ml. 43% had an MIC 4 µg/ml, 14% had an MIC 64 µg/ml and 43% with 128 µg/ml. Figure (8)

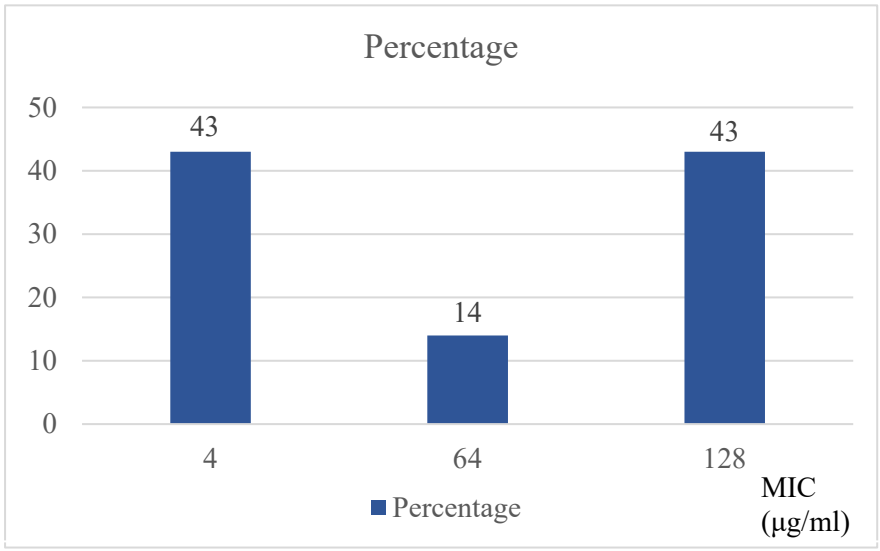

Figure 8. Percentages of Colistin's Minimum Inhibitory Concentration (MIC) of the *mcr-1*-positive-*E. coli* isolated from goat fecal samples

Finally, for the *E. coli* isolated from the cow, the MIC reported was 128 µg/ml. Moreover, the isolates expressed phenotypic resistance against Penicillin (100%), Ampicillin (63%), Amoxicillin/Clavulanic acid (96%), Cefepime (11%), Cefotaxime (37%), Cephalexin (96%), Cefixime (22%), Doripenem (0%), Meropenem (4%), Imipenem (0%), Gentamicin (33%), Kanamycin (30%), Streptomycin (41%), Tetracycline (44%), Ciprofloxacin (11%), Norfloxacin (11%), Trimethoprim/Sulfamethoxazole (11%) and Chloramphenicol (11%) as shown by the disc diffusion assay. Figure (9)

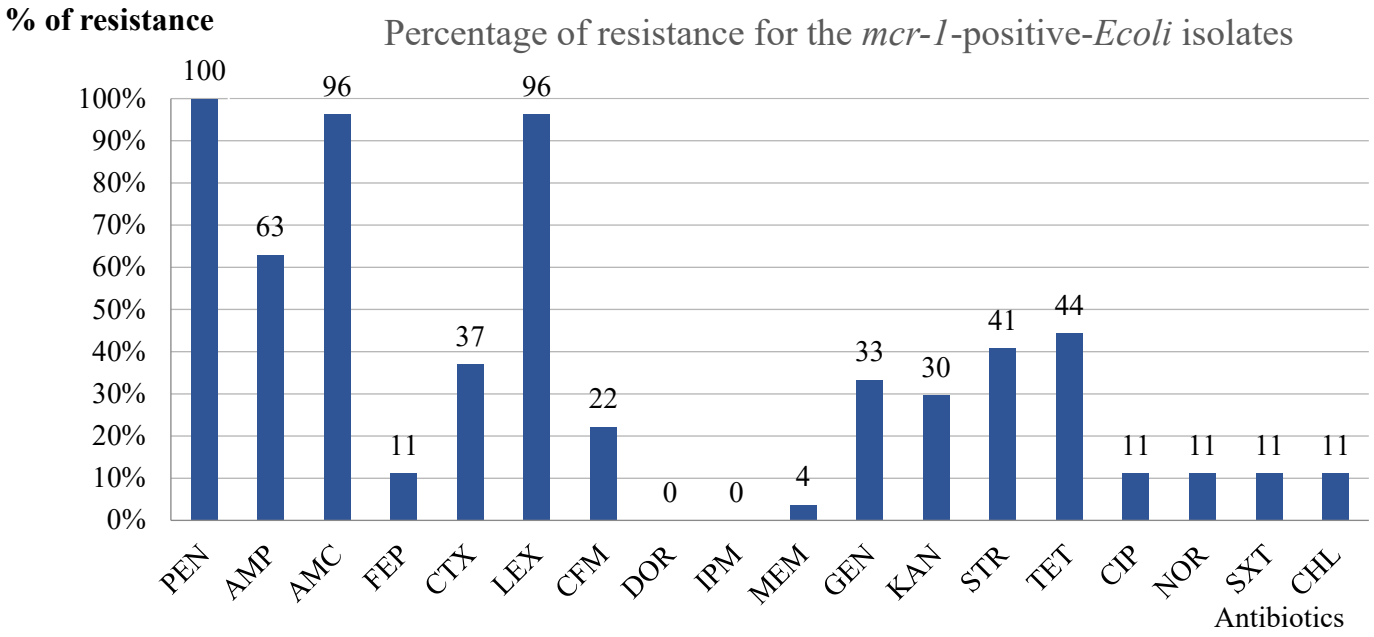

Figure 9. Percentages of resistance for the *mcr-1*-positve-*E. coli* isolates.

Interestingly, 81.5% of the isolates were multidrug-resistant, resisting at least three classes of antibiotics. It is noteworthy that 4% were both colistin and carbapenem-resistant, expressing resistance against meropenem.

The antimicrobial resistance profile for the *mcr-1* positive isolates originated from **sheep** showed phenotypical resistance against Penicillin (100%), Ampicillin (89%), Amoxicillin/Clavulanic acid (100%), Cefepime (0%), Cefotaxime (22%), Cephalexin (100%), Cefixime (11%), Doripenem (0%), Meropenem (0%), Imipenem (0%), Gentamicin (11%), Kanamycin (22%), Streptomycin (56%), Tetracycline (33%), Ciprofloxacin (0%), Norfloxacin (0%), Trimethoprim/Sulfamethoxazole (0%) and Chloramphenicol (0%). Figure (10)

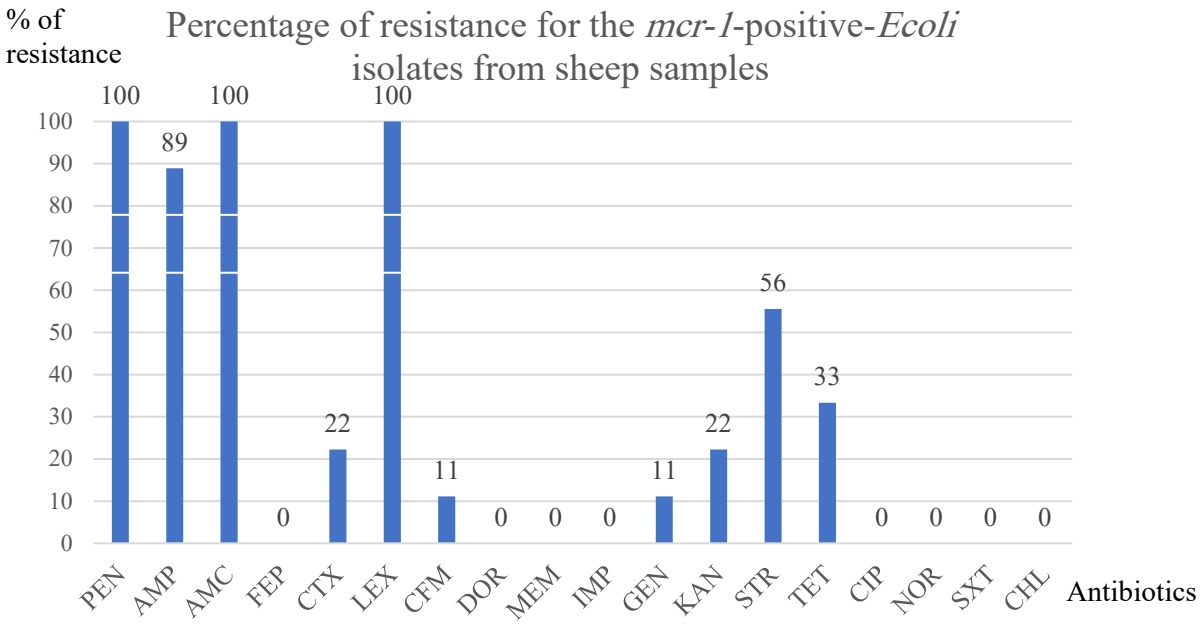

Figure 10. Percentages of resistance for the *mcr-1*-positve-*E. coli* isolates from sheep fecal samples.

For **Pigeons**, the resistance profile for the *mcr-1* positive isolates was as the following: Penicillin (100%), Ampicillin (71%), Amoxicillin/Clavulanic acid (71%), Cefepime (0%), Cefotaxime (71%), Cephalexin (100%), Cefixime (14%), Doripenem (0%), Meropenem (0%), Imipenem (0%), Gentamicin (57%), Kanamycin (29%),

Streptomycin (43%), Tetracycline (71%), Ciprofloxacin (43%), Norfloxacin (43%), Trimethoprim/Sulfamethoxazole (43%) and Chloramphenicol (43%). Figure (11)

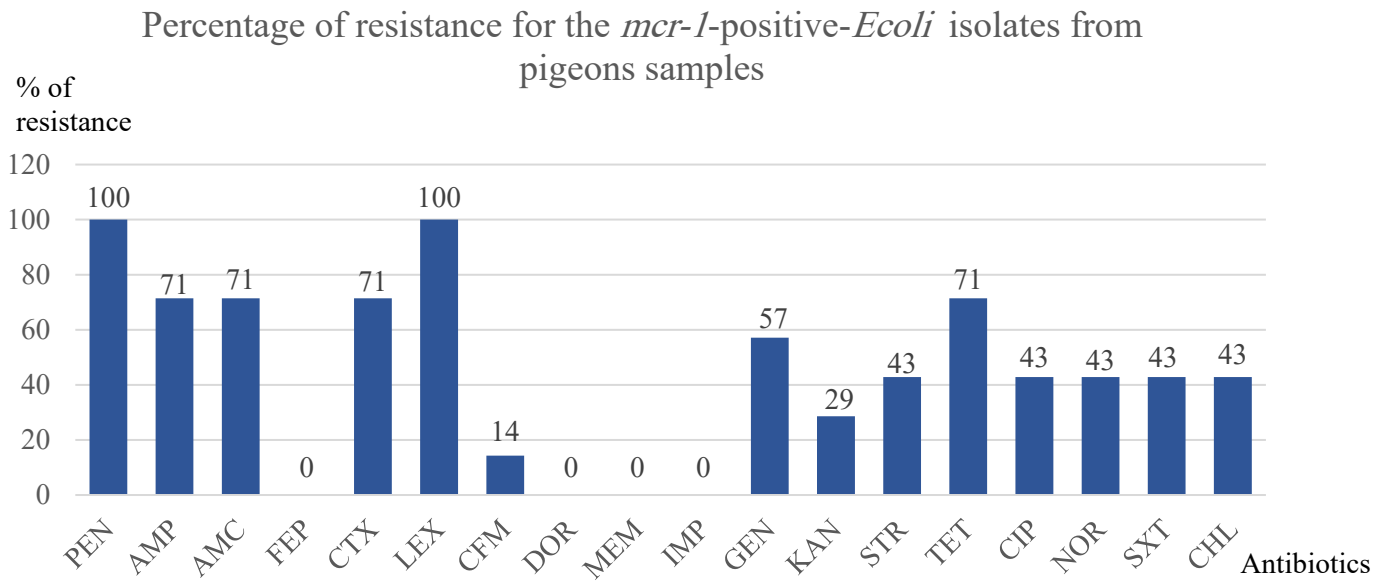

Figure 11. Percentages of resistance for the *mcr-1*-positve-*E. coli* isolates from pigeon fecal samples

The antimicrobial resistance profile for the *mcr-1* positive isolates originated from **chicken** samples showed phenotypical resistance against Penicillin (100%), Ampicillin (75%), Amoxicillin/Clavulanic acid (100%), Cefepime (50%), Cefotaxime (75%), Cephalexin (100%), Cefixime (75%), Doripenem (0%), Meropenem (25%), Imipenem (0%), Gentamicin (50%), Kanamycin (50%), Streptomycin (25%), Tetracycline (75%), Ciprofloxacin (0%), Norfloxacin (0%), Trimethoprim/Sulfamethoxazole (0%) and Chloramphenicol (0%). Figure (12)

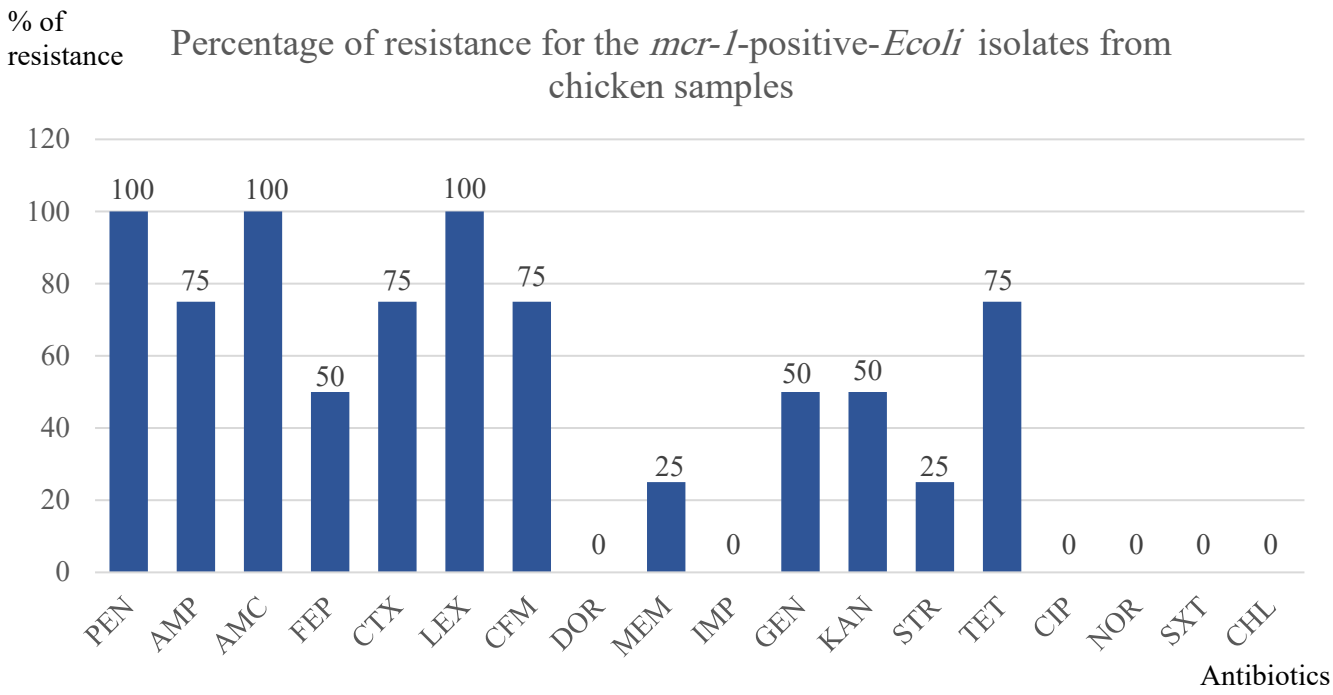

Figure 12. Percentages of resistance for the *mcr-1*-positve-*E. coli* isolates from chicken fecal samples

The antimicrobial resistance profile for the *mcr-1* positive isolates originated from **goat** samples showed phenotypical resistance against Penicillin (100%), Ampicillin (17%), Amoxicillin/Clavulanic acid (100%), Cefepime (17%), Cefotaxime (0%), Cephalexin (83%), Cefixime (0%), Doripenem (0%), Meropenem (0%), Imipenem (0%), Gentamicin (17%), Kanamycin (0%), Streptomycin (17%), Tetracycline (17%), Ciprofloxacin (0%), Norfloxacin (0%), Trimethoprim/Sulfamethoxazole (0%) and Chloramphenicol (0%). Figure (13)

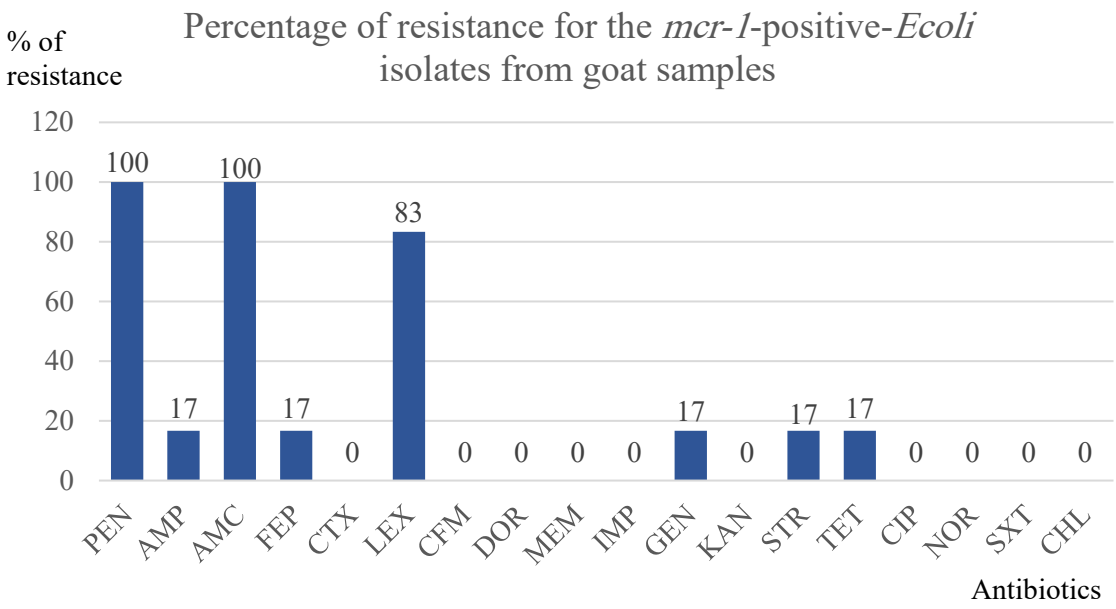

Figure 13. Percentages of resistance for the *mcr-1*-positve-*E. coli* isolates from goat fecal samples

Finally, for the one *mcr-1* positive isolate from the **cow** fecal samples showed phenotypical resistance against Penicillin, Amoxicillin/Clavulanic acid, Cefotaxime, Cephalexin, Gentamicin, and Kanamycin.

It is noteworthy that the class 1 integron gene presented in all the *mcr-1* positive isolates. Moreover, 44%, 71%, 75% of the sheep, pigeons and chickens co-carried *bla-TEM* gene respectively. Additionally, 25% of the *mcr-1* positive chicken samples harbored *bla-CTX.*

BOX PCR fingerprinting analysis revealed that the 27 *mcr-1* positive *E. coli* isolates were diverse and associated to different genotypes.

The *mcr-1* gene was successfully transformed into chemically competent *E. coli* JM109 recipient cells using the heat-shock method in all the colistin resistant isolates (n=27). All the transformants harbored the *mcr-1* gene, as shown by PCR

analysis, confirming the plasmid-borne nature of the gene. Also, colistin's MIC of the *E. coli* JM109. prior the transformation was < 0.25 µg/mL; however, the transformants' MIC varied between 2-32 µg/mL, thus confirming the colistin-resistant nature. Additionally, some of the transformants exhibited resistance to other antibiotics was observed, like resistance to Cephalosporins, Aminoglycoside, and Penicillins, suggesting that these genes may also be plasmid-borne as well.

Besides, multiple plasmid types were determined for 12 isolates from the mcr-*1* positive samples using the PBRT kit (Table 2). The recovered plasmid shows association with the global dissemination of *MCR* genes and other antibiotic resistance genes.

Table 2 The various plasmid types identified for the *mcr-1* positive isolates

Sample	Plasmid Type
Sheep 1	FIA-FIB-FII
Sheep 2	$I_{1\alpha}$ - X_2
Sheep 3	$I_{1\alpha}$ - FIA-FIB-FIBM
Pigeon 1	X_1
Pigeon 2	$I_{1\alpha}$ - FIB-FII
Pigeon 3	FIB-FIBM
Chicken 1	FIB-FIBM
Chicken 2	FIB-FIBM
Goat 1	X4-FII
Goat 2	FII
Goat 3	X1-FII
Cow 1	HI1-FII

12 amplified *mcr-1* gene fragments were purified using the PCR purification kit and sent out to be commercially sequenced. The isolates showed 100% homology to the previously reported *mcr-1* gene

*Table 4: summary of the analysis of the *mcr-1* positive *E. coli* isolates.

*: In the sample identification code, the numbers indicate different animals (Animal species), (C for cows), (G for Goats), (S for Sheep), (P for Pigeons), (Ch for Chicken) (The first number is for the animal number). Hence, Ch1 is chicken number one. (The second number is the isolate number). Therefore Ch1 1 is the isolate number one form the chicken number 1. Penicillin (PEN), ampicillin (AMP), amoxicillin+calvulanic acid (AMC), cefepime (FEP), cefotaxime (CTX), cephalexin (LEX), cefixime (CFM), doripenem (DOR),imipenem(IPM), meropenem (MEM), gentamicin (GEN), kanamycin (KAN), streptomycin (STR), tetracycline (TET), ciprofloxacin (CIP), norfloxacin (NOR), trimethoprim-sulfamethoxazole (SXT), and chloramphenicol (CHL). The antibiotics in the resistance profile were arranged according to the order of antibiotics/ classes listed in the CLSI guidelines.

Sample Identification Code	Resistance Profile	Intermediate Resistance Profile	Colistin MIC (µg/ml)	Genes
S1 1	PEN-AMP-AMC-CTX-LEX-CFM	GEN-KAN-STR-TET-	4	*Mcr-1*, *bla*TEM, Class 1 Integron genes.
S1 2	PEN-AMP-AMC-LEX-KAN-STR	FEP-GEN	16	*Mcr-1*, *bla*TEM, Class 1 Integron genes.
S1 3	PEN-AMP-AMC-LEX-STR-	FEP-CTX-CFM-DOR-MEM-KAN-	4	*Mcr-1*, Class 1 Integron genes.
S2 1	PEN-AMP-AMC-LEX-STR-	FEP-CTX-CFM-IPM-GEN-KAN-	8	*Mcr-1,* Class 1 Integron genes.
S2 2	PEN-AMP-AMC-LEX-	FEP-CTX-CFM-MEM-KAN-STR-	4	*Mcr-1*, Class 1 Integron genes.
S2 3	PEN-AMP-AMC-LEX-STR-TET-	FEP-CTX-CFM-DOR-MEM-IPM-GEN-KAN-	64	*Mcr-1*, *bla*TEM, ,Class 1 Integron genes.
S3 1	PEN-AMP-AMC-CTX-LEX-TET-	FEP-CFM-GEN-KAN-STR-	4	*Mcr-1*, Class 1 Integron genes.
S3 2	PEN-AMP-AMC-LEX-GEN-KAN-STR-TET-	FEP-CTX-IPM-	64	*Mcr-1*, Class 1 Integron genes.
S3 3	PEN-AMC-LEX-KAN-	FEP-CTX-MEM-GEN-STR-	4	*Mcr-1, bla*TEM, Class 1

				Integron gene s.
P3 1	PEN-LEX	FEP-CTX-GEN-KAN-TET	64	*Mcr-1*, *bla*TEM, Class 1 Integron genes.
P4 1	PEN-AMP-AMC-CTX-LEX-GEN-KAN-STR-TET-SXT-CHL-	FEP-CTX-IPM-KAN-STR-TET	16	*Mcr-1, bla*TEM, Class 1 Integron genes.
P4 2	PEN-AMP-AMC-LEX-CFM-GEN-KAN-TET-CIP-NOR-SXT-CHL	FEP-CFM-IPM	64	*Mcr-1*, *bla*TEM, Class 1 Integron genes.
P4 3	PEN-AMP-AMC-CTX-LEX-CFM-GEN-KAN-TET-CIP-NOR-SXT-CHL	FEP-MEM-	64	*Mcr-1, blaTEM,* Class 1 Integron genes.
P5 1	PEN-AMC-LEX-CFM-GEN-STR-TET-CIP-NOR-	AMP-FEP-DOR-MEM-IPM-KAN-	16	*Mcr-1, blaTEM,* Class 1 Integron genes.
P5 2	PEN-AMP-AMC-CTX-LEX-TET-CIP-NOR-	FEP-CFM-MEM-IPM-GEN-KAN-STR-	16	*Mcr-1*, Class 1 Integron genes.
P5 3	PEN-AMP-LEX-CFM-GEN-KAN-TET-CIP-NOR-CHL	CTX-MEM-KAN-STR-	8	*Mcr-1*, Class 1 Integron genes.
Ch1 1	PEN-AMC-CTX-LEX	AMP-FEP-GEN-KAN	16	*Mcr-1*, Class 1 Integron genes.
Ch5 1	PEN-AMP-AMC-FEP-CTX-LEX-CFM-GEN-KAN-STR-TET-	DOR-MEM-IPM-CIP-	1024	*Mcr-1*, *bla*TEM, *blaCTX-M,* Class 1 Integron genes.
Ch5 2	PEN-AMP-AMC-LEX-CFM-STR-TET-	FEP-CTX-KAN-	4	*Mcr-1, bla*TEM,, Class 1 Integron genes.
Ch5 3	PEN-AMP-AMC-FEP-CTX-LEX-CFM-MEM-GEN-KAN-TET	IPM-DOR	128	*Mcr-1*, *bla*TEM,, Class 1 Integron genes.
G1 1	PEN-AMP-AMC-LEX -GEN	FEP-CTX-DOR-MEM-KAN-STR	128	*Mcr-1,* Class 1 Integron genes.
G2 1	PEN-AMC-LEX	CTX-MEM-GEN-KAN-STR	4	*Mcr-1, Class 1 Integron genes.*

G2 2	PEN-AMC-LEX	AMP-FEP-CTX-IPM-KAN-STR	128	*Mcr-1, Class 1 Integron genes.*
G3 1	PEN-AMC-TET	AMP-FEP-CTX-CFM-IPM-KAN-STR	4	*Mcr-1, Class 1 Integron genes.*
G3 2	PEN-AMC-FEP-LEX-STR	CTX-IPM-KAN-STR	128	*Mcr-1, Class 1 Integron genes.*
G3 3	PEN-AMC-LEX	AMP-FEP-MEM-KAN	64	*Mcr-1, Class 1 Integron genes.*
C1 1	PEN-AMC-CTX-LEX-GEN-KAN	FEP-CFM-DOR-IPM-MEM-STR	128	*Mcr-1, Class 1 Integron genes.*

45 *E. Coli* colonies were isolated from the plates that had no colistin. the isolates expressed phenotypic resistance against Erythromycin (100%) ,Penicillin (100%), Ampicillin (13%), Amoxicillin/Clavulanic acid (51%), Cefepime (0%), Cefotaxime (2%), Cephalexin (44%), Cefixime (7%), Doripenem (0%), Meropenem (0%), Imipenem (2%), Gentamicin (2%), Kanamycin (2%), Streptomycin (7%), Tetracycline (22%), Ciprofloxacin (0%), Norfloxacin (0%), Trimethoprim/Sulfamethoxazole (4%) and Chloramphenicol (2%) as shown by the disc diffusion assay. Figure (14)

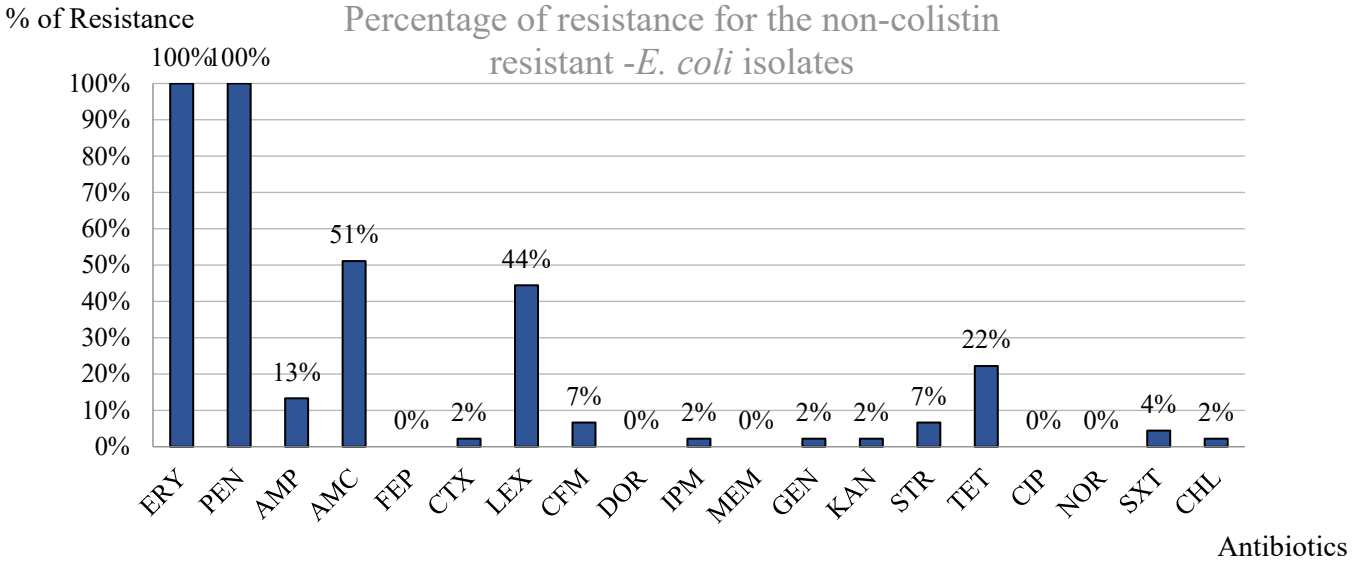

Figure 14. Percentages of resistance for the non-colistin resistance *E. coli* isolates

Its notable that, 15.5% of the isolates were multidrug-resistant, resisting at least three classes of antibiotics.

The antimicrobial resistance profile for the non-colistin isolates originated from **Pigeons** samples showed phenotypical resistance against Penicillin (100%), Ampicillin (11%), Amoxicillin/Clavulanic acid (0%), Cefepime (0%), Cefotaxime (0%), Cephalexin (11%), Cefixime (0%), Doripenem (0%), Meropenem (0%), Imipenem (0%), Gentamicin (11%), Kanamycin (0%), Streptomycin (0%), Tetracycline (55%), Ciprofloxacin (0%), Norfloxacin (0%), Trimethoprim/Sulfamethoxazole (11%) and Chloramphenicol (11%). Figure (15)

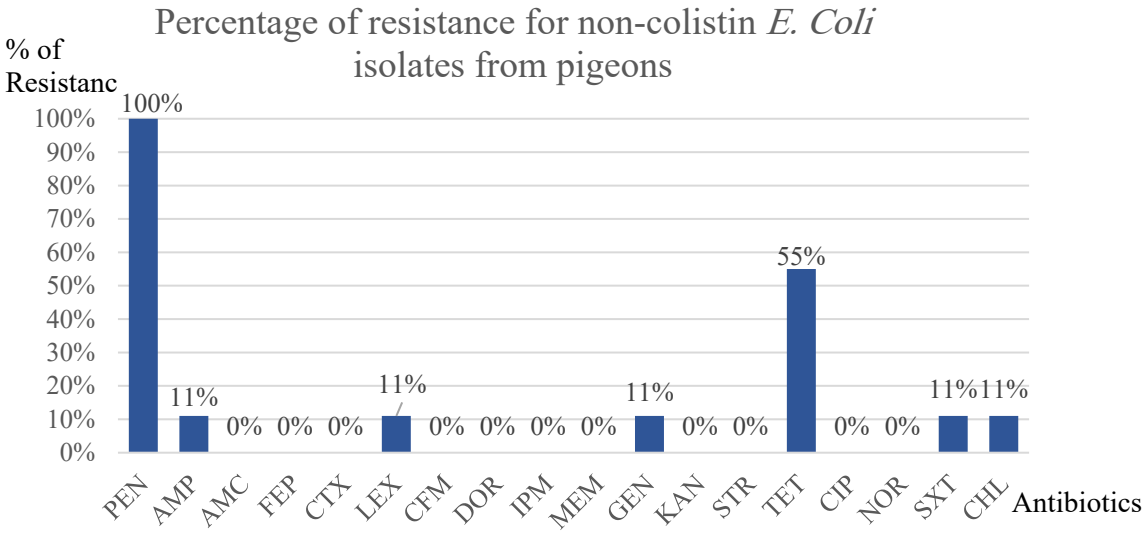

Figure 15. Percentages of resistance for the non-colistin resistance *E. coli* isolates from pigeon fecal samples.

For **chicken,** the non-colistin isolates showed phenotypical resistance against Penicillin (100%), Ampicillin (44%), Amoxicillin/Clavulanic acid (33%), Cefepime (0%), Cefotaxime (11%), Cephalexin (56%), Cefixime (0%), Doripenem (0%), Meropenem (0%), Imipenem (11%), Gentamicin (0%), Kanamycin (11%), Streptomycin (11%),

Tetracycline (44%), Ciprofloxacin (0%), Norfloxacin (0%),

Trimethoprim/Sulfamethoxazole (11%) and Chloramphenicol (0%). Figure (16)

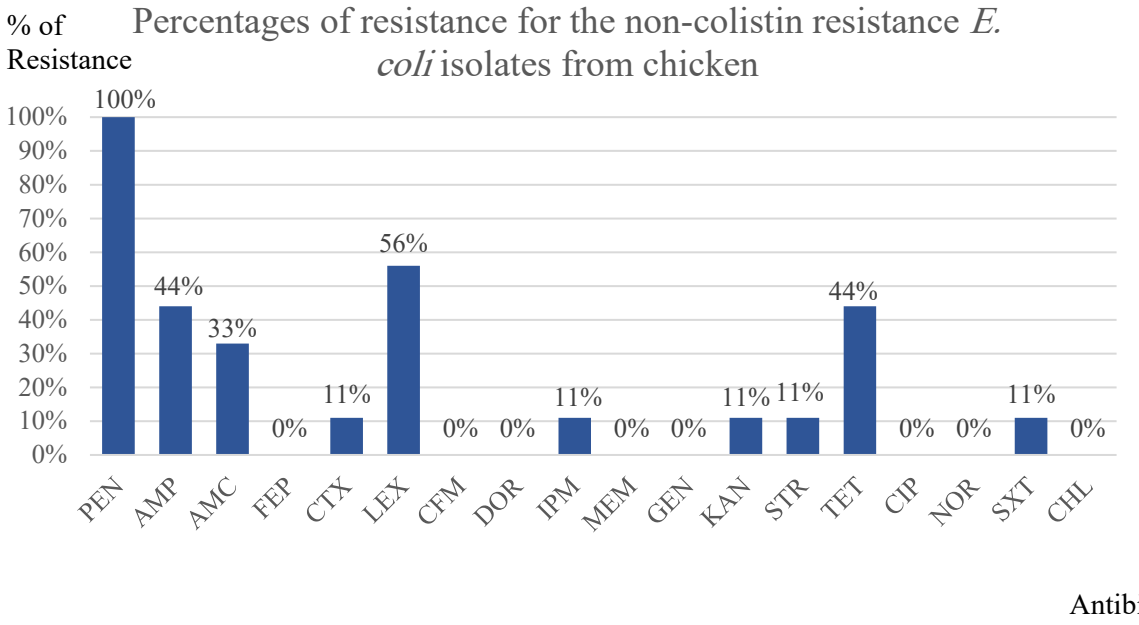

Figure 16. Percentages of resistance for the non-colistin resistance *E. coli* isolates from chicken fecal samples.

For **sheep,** the non-colistin isolates showed phenotypical resistance against Penicillin

(100%), Ampicillin (11%), Amoxicillin/Clavulanic acid (67%), Cefepime (0%),

Cefotaxime (0%), Cephalexin (56%), Cefixime (11%), Doripenem (0%), Meropenem

(0%), Imipenem (0%), Gentamicin (0%), Kanamycin (0%), Streptomycin (11%),

Tetracycline (11%), Ciprofloxacin (0%), Norfloxacin (0%),

Trimethoprim/Sulfamethoxazole (11%) and Chloramphenicol (0%). Figure (17)

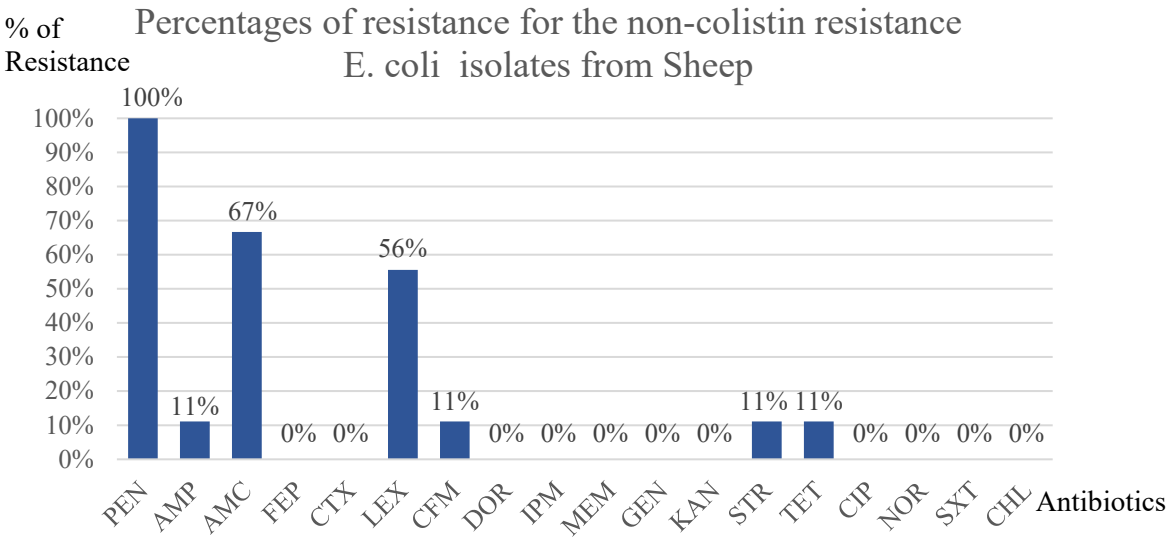

Figure 17. Percentages of resistance for the non-colistin resistance *E. coli* isolates from sheep fecal samples.

The antimicrobial resistance profile for the non-colistin isolates originated from **goats** samples revealed phenotypical resistance against Penicillin (100%), Ampicillin (0%), Amoxicillin/Clavulanic acid (100%), Cefepime (0%), Cefotaxime (0%), Cephalexin (44%), Cefixime (11%), Doripenem (0%), Meropenem (0%), Imipenem (0%), Gentamicin (0%), Kanamycin (0%), Streptomycin (11%), Tetracycline (11%), Ciprofloxacin (0%), Norfloxacin (0%), Trimethoprim/Sulfamethoxazole (0%) and Chloramphenicol (0%). Figure (18)

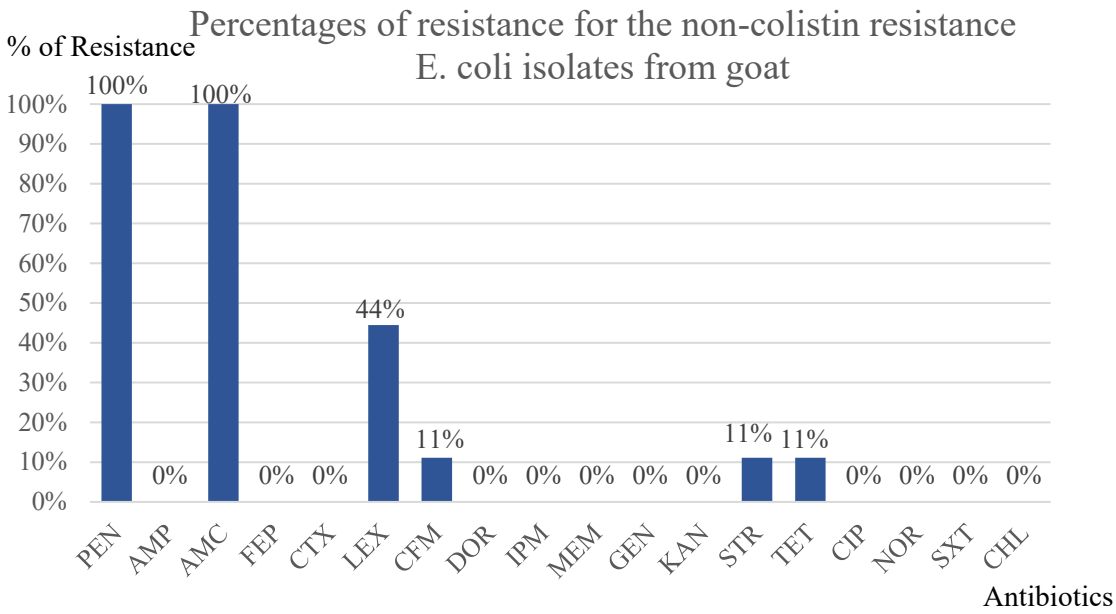

Figure 18. Percentages of resistance for the non-colistin resistance *E. coli* isolates from goat fecal samples.

For **cow ,** the non-colistin isolates showed phenotypical resistance against Penicillin (100%), Ampicillin (0%), Amoxicillin/Clavulanic acid (67%), Cefepime (0%), Cefotaxime (0%), Cephalexin (44%), Cefixime (11%), Doripenem (0%), Meropenem (0%), Imipenem (0%), Gentamicin (0%), Kanamycin (0%), Streptomycin (0%), Tetracycline (0%), Ciprofloxacin (0%), Norfloxacin (0%), Trimethoprim/Sulfamethoxazole (0%) and Chloramphenicol (0%). Figure (19)

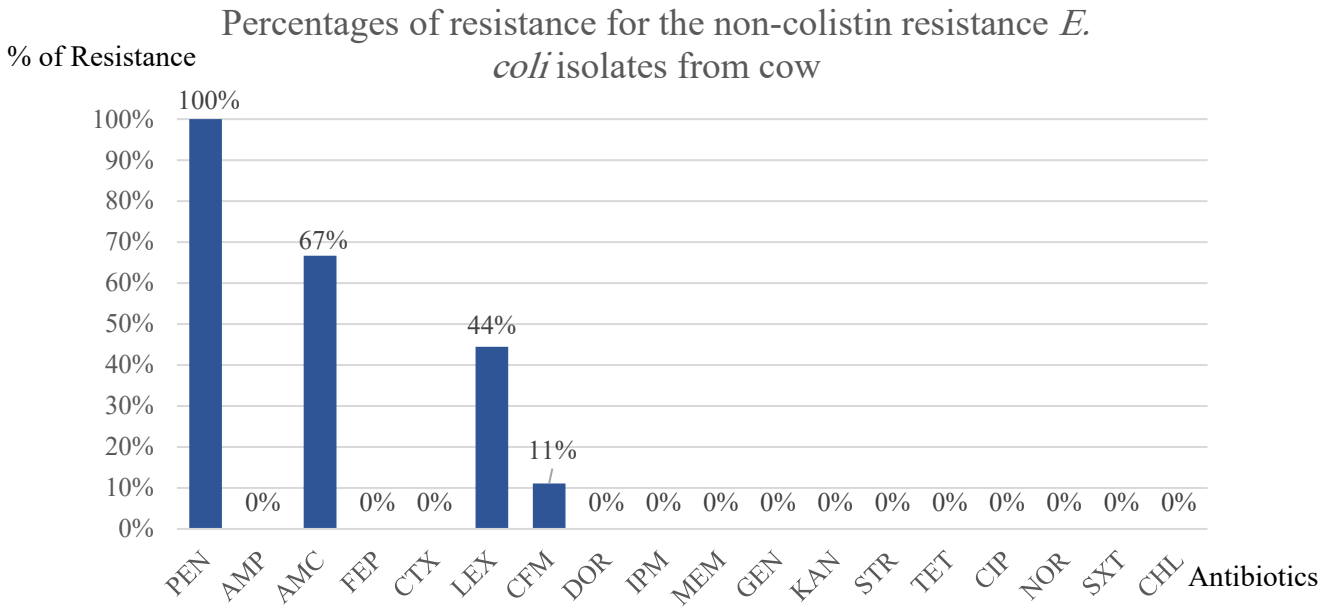

Figure 19. Percentages of resistance for the non-colistin resistance *E. coli* isolates from cow fecal samples.

It is noteworthy that the class 1 integron gene presented in 95.5% of the non-colistin *E. coli* isolates. Moreover, 55.5%, 11%, 11% of the non-colisitn isolates from chicken, sheep and goats carried *bla-TEM* gene respectively.

CHAPTER 4

DISCUSSION

Antimicrobial resistance (AMR) affects millions of people worldwide, with notable severe implications on food safety, social development and the world economy (57). The emergence of multidrug-resistant pathogens prompted the reintroduction of colistin, as a last-resort antibiotic, into clinical practice (58). However, the uncontrolled use of colistin in animal farming drove the emergence of colistin resistance, initially believed to be chromosomal (59). However, the discovery of a mobile plasmid-borne colistin-resistance gene, *mcr-1*, revealed horizontal transmission between bacteria, further complicating the spread of resistance (50).

The "One Health" approach states that the health of humans and animals are interconnected. An increasing body of evidence suggests that backyard animals, particularly those in environments with inadequate sanitation in economically challenged countries such as Lebanon, may serve as reservoirs for antibiotic-resistant organisms and genes.

In Lebanon, previous studies reported the widespread presence of the *mcr-1* gene in various environments across Lebanon, including irrigation and seawater, pre-harvest poultry, and Syrian refugee camps (60). Consequently, it became essential to investigate the prevalence of the *mcr-1* gene in a different unstudied niche such as backyard animal farms in Lebanon.

All *E. coli* isolates resistant to colistin carried *mcr-1*, while 81.5% of the isolates were multidrug-resistant, resisting at least three classes of antibiotics, and 4% were both colistin and carbapenem-resistant, expressing resistance against meropenem. These

results pose a significant issue as colistin is administered as a last-resort treatment in cases where cephalosporins and carbapenems are ineffective. Furthermore, the *bla-$_{TEM}$* gene was co-carried by 44% of isolates from sheep, 71% of pigeons, and 75% of chickens. Among the *mcr-1* positive isolates from chicken samples, 25% also harbored the *bla-$_{CTX}$* gene.

The presence of the Class 1 Integron gene was documented in 100% of the isolates, underscoring the ability of these bacterial strains to evolve and acquire various resistance genes.

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The occurrence of multidrug-resistant (MDR) *E. coli* carrying the *mcr-1* gene potentially underscores the impact of environmental pollution and the misuse antibiotics in both humans and animals in Lebanon. This also underscores the potential transmission of these organisms to various environments, particularly via pigeons. Given their ability to fly between locations, pigeons may contribute to cross-contamination over extensive areas, affecting other animals, both domesticated and wildlife, as well as crucial resources like water and crops.

The *mcr-1* gene in the backyard isolates was plasmid-borne, raising concerns, because there's a risk of it transferring to other bacteria, potentially including pathogenic strains. It's important to emphasize that the *mcr-1* worldwide spread is significantly influenced by and the transmissibility of IncX4 plasmids across the globe.

BOX PCR fingerprinting analysis demonstrated the diversity among the 27 *mcr-1* positive *E. coli* isolates, which included potentially different genotypes.

In comparison, *E. coli* isolated from non-colistin containing media showed that 15.5% of the isolates exhibited multidrug resistance. It is also remarkable that the class 1

Integron gene was present in 95.5% of these isolates, highlighting the capability of these bacterial strains to potentially acquire and express diverse resistance genes. Additionally, 55.5%, 11%, and 11% of these isolates from chickens, sheep, and goats, respectively, carried the *bla-TEM* gene.

It is clear that the resistance in the "non-colistin" isolates was less than that of the colistin-resistant isolates. The discovery of *mcr-1* in animals in backyards presents a major public health risk in Lebanon. It is essential to adopt a comprehensive One Health strategy, enhance the control of antimicrobials, and prohibit the application of colistin in animal agriculture to address the consequences of antibiotic resistance on human health. In addition, there is a need to develop policies to control backyard farming and help the small-scale farmers by giving proper resources and education. Finally, more sampling must be done to have a better understanding of the prevalence of this gene in these animals. Moreover, advanced techniques like whole genome sequencing should be used to asses the genetic properties of the *mcr-1*-positive strains, including the resistome, virulome and plasmid and sequence types.

CHAPTER 5

CONCLUSION

Antimicrobial resistance is a worrisome and significant challenge in our ongoing era, as even Colistin, a last-resort antibiotic, is being reconsidered for the treatment of multidrug infections in clinical settings. However, the global dissemination of the *mcr-1* gene presents a serious threat to its efficacy. Unfortunately, countries suffering from economic difficulties and inadequate infrastructure and policies, such as Lebanon, face challenges in addressing the spread of *mcr* genes in humans, animals, and environmental samples, reflecting the situation in the MENA region. The lack of adequate studies on the dissemination of *mcr* genes in these countries could transform them into vital reservoirs for the global transmission of this gene. In Lebanon, the identification of the *mcr-1* gene in humans, animals, and environmental samples highlights its widespread existence in a country marked by weak antimicrobial stewardship and unregulated use of colistin in agriculture.

In our study, we focused on a previously unexplored environmental factor, the backyard animal farms. These farms serve as a direct food source for a significant population of the Lebanese families who view it as a safe and organic option. Additionally, the manure from these animals is widely utilized in agriculture. Our findings reported the presence of multidrug-resistant bacteria carrying the *mcr-1* gene in the targeted backyard, as a first report in Lebanon, this indicates the need for further investigations into this environmental factor. Urgent intervention is required to control the *mcr-1* dissemination in backyard animals and its potential cross-contamination with other environmental resources and humans. Consequently, addressing colistin resistance necessitates more studies on *mcr-1* in more environmental samples. This should be

coupled with the adoption of a one-health approach and the implementation of policies restricting colistin use in animal farming for growth promotion. Additionally, developing policies to regulate backyard farming and providing proper education for farmers is essential.

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